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Arsenite-oxidizing and arsenate-reducing bacteria associated with arsenic-rich groundwater in Taiwan

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ABSTRACT

Drinking highly arsenic-contaminated groundwater is a likely cause of blackfoot disease in Taiwan, but microorganisms that potentially control arsenic mobility in the subsurface remain unstudied. The objective of this study was to investigate the relevant arsenite-oxidizing and arsenate-reducing microbial community that exists in highly arsenic-contaminated groundwater in Taiwan. We cultured and identified arsenic-transforming bacteria, analyzed arsenic resistance and transformation, and determined the presence of genetic markers for arsenic transformation. In total, 11 arsenic-transforming bacterial strains with different colony morphologies and varying arsenic transformation abilities were isolated, including 10 facultative anaerobic arsenate-reducing bacteria and one strictly aerobic arsenite-oxidizing bacterium. All of the isolates exhibited high levels of arsenic resistance with minimum inhibitory concentrations of arsenic ranging from 2 to 200 mM. Strain AR-11 was able to rapidly oxidize arsenite to arsenate at concentrations relevant to environmental groundwater samples without the addition of any electron donors or acceptors. We provide evidence that arsenic-reduction activity may be conferred by the *ars* operon(s) that were not amplified by the designed primers currently in use. The 16S rRNA sequence analysis grouped the isolates into the following genera: *Pseudomonas*, *Bacillus*, *Psychrobacter*, *Vibrio*, *Citrobacter*, *Enterobacter*, and *Bosea*. Among these genera, we present the first report of the genus *Psychrobacter* being involved in arsenic reduction. Our results further support the hypothesis that bacteria capable of either oxidizing arsenite or reducing arsenate coexist and are ubiquitous in arsenic-contaminated groundwater.

1. Introduction

Arsenic (As), a known human carcinogen, is widely distributed in food, water, soils, and air. It is a ubiquitous element of both natural and anthropogenic origin and is often responsible for contaminating water supplies. In most cases, arsenic contamination of groundwater is derived naturally from arsenic-rich aquifer sediments. The two biologically relevant oxidation states of inorganic arsenic are arsenite [As(III)] and arsenate [As(V)]. In general, As(III) is more hazardous to organisms than As(V) (Hughes, 2002).

Certain microorganisms have evolved the necessary genetic components which confer resistance mechanisms, allowing them to survive and grow in environments containing levels of arsenic that would be toxic to most other organisms. In bacteria, high-level resistance to arsenic is conferred by the arsenical resistance (*ars*) operon. The bacterial *ars* operon is comprised of three to five genes (*arsR*, *-D*, *-A*, *-B*, and *-C*), which are located in plasmids (Owolabi and Rosen, 1990) or chromosomes (Diorio et al., 1995). *arsR* and *arsD* are regulatory genes, while *arsA* and *arsB* form a transmembrane efflux pump that exports As(III) from

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the cytoplasm, thus lowering intracellular concentrations of toxic arsenic (Mukhopadhyay et al., 2002). The *arsC* gene encodes the enzyme for As(V) reductase, which is responsible for the biotransformation of As(V) to As(III). In addition to detoxification mechanisms, some bacteria are known to possess mechanisms for either oxidizing As(III) or reducing As(V), including transformation linked to energy generation (Oremland and Stolz, 2005). As(V) respiratory reductase (*arr*) genes encode periplasmic As(V) reductase that functions in anaerobic respiration using As(V) as a terminal electron acceptor, while *aox* genes encode periplasmic As(III) oxidase that transforms As(III) to As(V) (Silver and Phung, 2005). Recently, a novel arsenite oxidase gene, *arxA*, was identified in the genome sequence of the Mono Lake isolate, *Alkalilimnicola ehrlichii* MLHE-1, a chemolithoautotroph that couples arsenite oxidation to nitrate reduction (Zargar et al., 2010).

Several studies successfully used genetic markers to study arsenic transformation mechanisms, such as *arsB* and *arsC* genes in the *ars* operon for arsenic resistance (Achour et al., 2007; Sun et al., 2004), the *arrA* gene for dissimilatory As(V) respiration (DAsR) (Kulp et al., 2007; Malasarn et al., 2004; Song et al., 2009), and the *aoxB* gene for As(III) oxidation (Hamamura et al., 2009; Rhine et al., 2007). However according to several studies, no *ars* genes were found in strains categorized within the same genera (Cai et al., 1998; Chang et al., 2007; Saltikov and Newman, 2003; Saltikov et al., 2003). Moreover, several studies observed that despite clear evidence of the As-transforming activity by microorganisms, no amplicon for arsenite oxidase (*aoxB*) or As(V) respiratory reductase (*arrA*) was attained using the reported polymerase chain reaction (PCR) primers and protocols (Handley et al., 2009; Kulp et al., 2008; Song et al., 2009).

Natural arsenic in groundwater used as a source for drinking water represents a significant human health problem which occurs most notably in parts of Bangladesh (Tareq et al., 2003), West Bengal (Nag et al., 1996), China (Sun, 2004), the United States (Robertson, 1989), and Taiwan (Lu, 1990). In Taiwan, the southwestern coast area is historically associated with endemic cases of blackfoot disease (BFD), a peripheral vascular disease caused by the long-term ingestion of arsenic-contaminated groundwater. In addition to skin pigmentation, a very high incidence of keratoses, and skin, lung, bladder, and other cancers was found among people who lived in the southwestern coastal area and who drank naturally occurring arsenic-contaminated well water prior to the 1990s (Chen et al., 1986; Huang et al., 2003; Tseng, 1977). At present, although well water is not directly ingested by most residents in the area, it is still extensively used to meet domestic, irrigation, aquacultural, and industrial needs. High bioaccumulated arsenic concentrations in farmed fish were associated with arsenic concentrations in pond waters in this area (Huang et al., 2003). In addition to the historical BFD region, high arsenic levels of groundwater were also found in several other regions of Taiwan, including Ilan, Yunlin, Chiayi, and Tainan Counties. Therefore, there is an urgent need to develop low-cost, efficient technologies to clean arsenic from groundwater in these high-arsenic regions. However, there is still an intense debate on the etiology of BFD as well as the sources of arsenic. Despite considerable research over the past 30 years into the occurrence and distribution of arsenic in the region's groundwater, few studies were conducted to investigate the geochemical and microbiological processes that influence the element's speciation and mobility in this aquifer. To this end, it is important to understand all relevant abiotic and biotic factors that contribute to arsenic mobility and sequestration in such environments.

It is well established that arsenic redox cycling by microorganisms plays a significant role in controlling arsenic speciation and mobility in high-arsenic environments (Oremland and Stolz, 2003, 2005). Microbial species with arsenic-biotransforming capabilities have so far not been investigated in arsenic-contaminated regions of Taiwan. The objective of this study was to investigate the relevant As(III)-oxidizing and As(V)-reducing microbial communities that exist in highly arsenic-contaminated groundwater in Taiwan. Such studies will improve our understanding of the mobilization and immobilization of arsenic in such environments and help us better understand how microbes metabolize arsenic. We cultured and identified arsenic-transforming bacteria, analyzed arsenic resistance and transformation, and determined the presence of genetic markers for arsenic transformation. Results of this study will provide valuable information on microbial species that mediate arsenic redox transformations that may contribute to the release of sedimentary arsenic to groundwater in the region.

2. Materials and methods

2.1. Site description and sample collection

The arsenic level of groundwater is high (0.12 ± 0.14 mg/L) in shallow monitoring wells of the Choushui River alluvial fan (Wang et al., 2007) which includes Yunlin County, west-central Taiwan. Arsenic-contaminated groundwater samples were collected from well AG1 (20 m in depth) in southern Yunlin County, which is close to coastal and vicinal BFD hyperendemic areas, an area known to have elevated concentrations of arsenic present in shallow groundwater (Fig. 1). Sample pH values ranged from 7.0 to 7.6, and temperatures varied from 22 to 27 °C.

2.2. Isolation and growth of arsenic-resistant bacteria

Groundwater samples were inoculated into a chemically defined medium (CDM) (8.12 mM $MgSO_4$, 18.7 mM NH_4Cl, 7 mM Na_2SO_4, 0.0574 mM K_2HPO_4, 0.457 mM $CaCl_2$, 44.6 mM Na-lactate, 0.012 mM Fe_2SO_4, and 9.5 mM $NaHCO_3$, with the pH adjusted to 7.2) as described by Weeger et al. (1999) to enrich bacterial populations with or without 2 mM of As(III) or 10 mM of As(V) under oxic and anoxic incubation, respectively. Samples were then incubated at 25 °C for 24 h. After incubation, samples were serially diluted, and a cell suspension was spread onto CDM plates with or without 2 mM of As(III) or 10 mM of As(V) to obtain single colonies. A pure culture was obtained by successive isolation of colonies at 25 °C in As(III)- or As(V)-supplemented medium. Bacterial cultures were incubated on a rotary shaker or placed in an anaerobic chamber (Ruskinn Technology, Leeds, UK) for oxic and anoxic incubation, respectively. Resistance to arsenic was defined as the ability of bacteria to grow on CDM agar plates containing either 2 mM As(III) or 10 mM As(V) at 25 °C.

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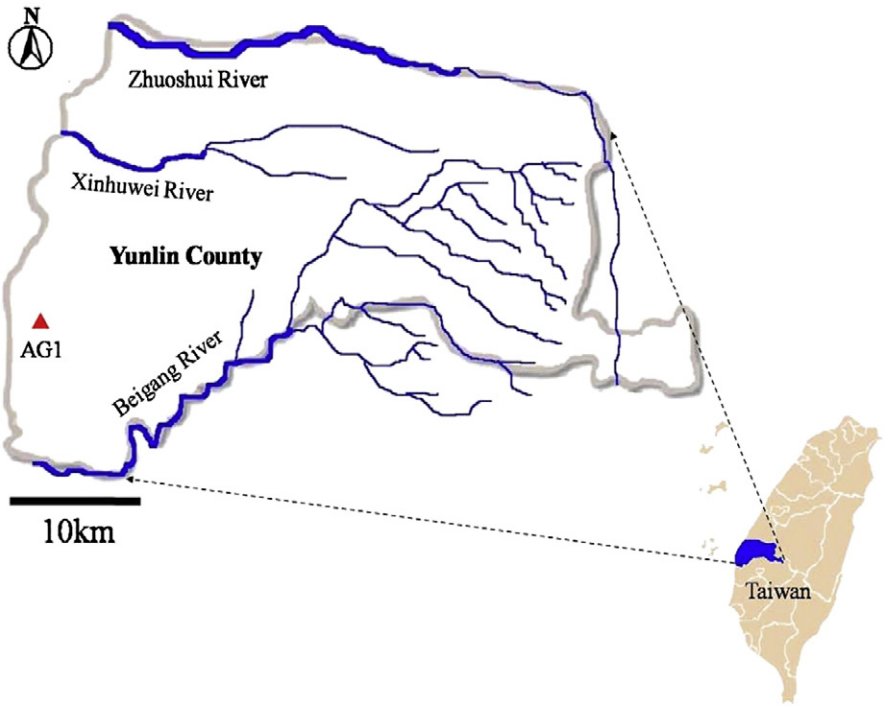

Fig. 1. Site map of the study area and approximate sampling site (▲). Arsenic-contaminated groundwater was collected from well AG1.

2.3. Arsenic-transformation assay

The abilities of the obtained arsenic-resistant bacteria to oxidize As(III) and/or reduce As(V) were tested under both aerobic and anaerobic conditions. Aerobic cultures were incubated with constant shaking. Anaerobic cultures were placed in an anaerobic chamber. The isolates were first tested using a qualitative $AgNO_3$ screening method as described by Simeonova et al. (2004) with slight modification. Briefly, stationary-phase culture was centrifuged and then washed twice with minimal salt medium (MSM) (Macy et al., 2000). The collected bacterial pellets were resuspended in MSM containing 10 mM glucose and either 1 mM As(III) or As(V). The bacterial suspensions (with an optical density at 600 nm of 0.3, $OD_{600} = 0.3$) were incubated at room temperature for 72 h. Subsequently, the bacterial cultures were centrifuged, and 100 μL of the liquid phase was mixed with 100 μL of a 0.1 M $AgNO_3$ solution. The resulting precipitates containing arsenic were colored from light yellow of Ag_3AsO_3 (silver orthoarsenite) due to As(III) to light brown–red of Ag_3AsO_4 (silver orthoarsenate) due to As(V).

The abilities of the bacterial isolates to oxidize As(III) and/or reduce As(V) were further quantitatively determined. Bacterial isolates were cultured as described for the $AgNO_3$ screening method. After 72 h of incubation, the bacterial cultures were centrifuged, and arsenic species in the supernatants were measured by a molybdenum blue spectrophotometric assay (Johnson and Pilson, 1972).

We also used the As(V)-respiring strain, *Shewanella* sp. strain ANA-3 (a kind gift from Dr. Dianne Newman, Massachusetts Institute of Technology, Cambridge, MA, USA), as a reference strain for the As(V) reduction rate assay. Strains AR-7 and ANA-3 were cultured as described above, and the bacterial suspensions ($OD_{600} = 0.3$) were incubated in MSM containing 10 mM glucose and 0.25 mM As(V) under aerobic or anaerobic conditions. Strain AR-11 was cultured as described above, and the bacterial suspension ($OD_{600} = 0.3$) was incubated in MSM containing 10 mM glucose and 0.25 mM As(III) under aerobic conditions. Samples were periodically withdrawn, and the arsenic species were measured.

Additionally, the ability of strain AR-11 to transform arsenic at concentrations relevant to the environmental groundwater was tested. Strain AR-11 was cultured and assayed as described above except that the medium was replaced with an environmental groundwater sample that was previously autoclaved until it was sterile without the addition of any electron donors or acceptors. The collected environmental groundwater contained 780 μg L^{-1} As(III).

2.4. Determination of minimum inhibitory concentrations (MICs)

As(III) and As(V) resistance of the bacterial isolates was evaluated by MIC tests under both aerobic and anaerobic conditions. MICs of the isolated strains were assessed as previously described (Achour et al., 2007; Escalante et al., 2009; Lim and Cooksey, 1993). Aliquots of bacterial cultures ($OD_{600} = 0.3$) were grown on MSM agar plates containing different concentrations of As(III) (2–20 mM) or As(V) (2–200 mM) and then incubated at 25 °C in oxic or anoxic conditions. The MIC is defined as the lowest concentrations of As(III) or As(V) that completely inhibited bacterial growth on agar medium after 72 and 120 h of incubation for aerobic and anaerobic growth conditions, respectively.

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2.5. Physiological characterization of the strains

The abilities of strains AR-4, AR-5, and AR-11 to grow under a range of temperatures and pH values on CDM medium were examined. Strains AR-4, AR-5, and AR-11 were further characterized using the API ZYM system for an enzyme analysis and the API 50CH system for a carbohydrate-utilization analysis following the manufacturer's instructions (bioMérieux Vitek, Hazelwood, MO, USA).

2.6. Isolation of genomic DNA

Bacterial genomic DNA was prepared as described by Wilson (1997) with slight modification. Briefly, bacterial pellets were collected from 1.5 mL of bacterial cultures, and resuspended in 564 μL of Tris–ethylenediaminetetraacetic acid (EDTA) buffer (pH 8.0), 30 μL of 10% sodium dodecylsulfate (SDS), and 6 μL of 10 mg/mL proteinase K, and incubated at 37 °C for 1 h. The solution was mixed with 100 μL of 5 M NaCl, and then mixed thoroughly with 80 μL of a NaCl/cetyltrimethyl ammonium bromide (CTAB) solution. The solution was incubated at 65 °C for 10 min. Genomic DNA was extracted with chloroform/isoamyl alcohol (1:1) and phenol/chloroform/isoamyl alcohol (25:24:1). DNA was precipitated with an equal volume of isopropanol, washed with 75% ethanol, and resuspended in 50 μL of distilled H_2O.

2.7. PCR amplification of 16S ribosomal (r)RNA and arsenic-related marker genes

The PCR amplification of 16S rRNA and arsenic marker genes was performed using bacterial genomic DNA as a template. Sequences of the PCR primers for 16S rRNA and arsenic marker genes are presented in Table 1. Universal primers were used to amplify 16S rRNA. Degenerate primers used to amplify the arsenic marker genes were respectively designed especially for *arsB*, *arsC*, *arrA,* and *aoxB*. The PCR conditions followed descriptions in the corresponding literature (Table 1). The amplified PCR products were purified using a BioMan kit (Taipei, Taiwan). The presence of 16S rRNA, *arsB*, *arsC*, *arrA*, and *aoxB* genes in the PCR product was confirmed by sequencing with an ABI Prism 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA).

2.8. Analysis of sequence data

The NCBI BLAST program was used to support the 16S rRNA generic identification of isolates and amplified arsenic marker gene sequences. For the phylogenetic analysis, 16S rRNA sequences of the bacterial isolates and related reference sequences retrieved from the GenBank database were aligned using ClustalX software (Thompson et al., 1997). Distance analysis and phylogenetic tree construction were performed using the neighbor-joining (NJ) method of *MEGA* vers. 4 (Tamura et al., 2007). In total, 1000 bootstrap replications were calculated. The tree was unrooted.

2.9. Sequence accession numbers

Sequences obtained in this study were deposited in the GenBank database. The accession numbers for the 16S rRNA nucleotide sequences are: FJ888371 (strain AR-1), FJ888372 (strain AR-2), FJ888373 (strain AR-3), FJ888374 (strain AR-4), FJ888375 (strain AR-5), FJ888376 (strain AR-6), FJ888377 (strain AR-7), FJ888378 (strain AR-8), FJ888379 (strain AR-9), FJ888380 (strain AR-10), and FJ888381 (strain AR-11).

3. Results

3.1. Isolation of arsenic-resistant bacteria

Well AG1 has been actively monitored for arsenic contamination by the Taiwan Industrial Development Bureau of the Ministry of Economic Affairs for the past 15 years. The well was reported to principally be contaminated with arsenic concentrations ranging from 0.3 to 0.78 mg/L. In total, 11 arsenic-transforming bacterial strains with different colony morphologies and varying arsenic-transformation abilities were isolated, including 10 facultative anaerobic As(V)-reducing bacteria and one strictly aerobic As(III)-oxidizing

Table 1
Oligonucleotide primers used in this study.

Targeted gene	Primer	Primer sequences (5′→3′)	Reference
16S	16S-F	GGAGCAAACAGGATTAGATACC	Keis et al. (1995)
	16S-R	TGCCAACTCTATGGTGTGTGACG	
arsB	darsB1-F	GGTGTGGAACATCGTCTGGAAYGCNAC	Achour et al. (2007)
	darsB1-R	CAGGCCGTACACCACCAGRTACATNCC	
arsC	amlt42-F	TCGCGTAATACGCTGGAGAT	Sun et al. (2004)
	amlt376-R	ACTTTCTCGCCGTCTTCCTT	
arrA	arrA-F	AAGGTGTATGGAATAAAGCGTTTGTBGGHGAYTT	Malasarn et al. (2004)
	arrA-R	CCTGTGATTTCAGGTGCCCAYTYVGGNGT	
arrA	haarrAD1-F	CCGCTACTACACCGAGGGCWWYTGGGRNTA	Kulp et al. (2007)
	haarrAG2-R	CGTGCGGTCCTTGAGCTCNWDRTTCCACC	
arrA	as1-F	CGAAGTTCGTCCCGATHACNTGG	Song et al. (2009)
	as1-R	GGGGTGCGGTCYTTNARY TC	
	as2-F	GTCCCNATBASNTGGGANRARGCN MT	
	as2-R	ATANGCCCARTGNCCYTGNG	
aoxB	69-F	TGYATYGTNGGNTGYGGNTAYMA	Rhine et al. (2007)
	1374-R	TANCCYTCYTGRTGNCCNCC	
aroA	aroA95-F	TGYCABTWCTGCAIYGYIGG	Hamamura et al. (2009)
	aroA599-R	TCDGARTTGTASGCIGGICKRTT	

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bacterium (Tables 2 and 3). Isolation of these bacterial strains was possible on the basis of their ability to grow in the presence of 2 mM As(III) or 10 mM As(V) and according to the criteria of different colony morphologies. All 10 bacterial isolates displayed facultative anaerobic growth, except for AR-11 which strictly featured aerobic growth (Tables 2 and 3).

3.2. Phenotypic characterization

Tolerances of the 11 bacterial isolates to As(III) and As(V) were assessed by MIC tests under aerobic and anaerobic growth conditions. All of the isolates were resistant to As(III) or As(V). Under aerobic growth conditions, all of the bacterial isolates exhibited resistance to As(V) with MICs exceeding 100 mM (Table 2). Six isolates (AR-3, AR-4, AR-6, AR-8, AR-9, and AR-10) were even resistant to As(V) at 200 mM (Table 2). MICs (up to 5 mM) for As(III) were found for five bacterial strains (AR-5, AR-7, AR-8, AR-9, and AR-10), while the remaining strains showed MICs to As(III) of 2.0–2.5 mM (Table 2). Under anaerobic growth conditions, the bacterial isolates showed different degrees of arsenic sensitivity. Five isolates (AR-2, AR-7, AR-8, AR-9, and AR-10), in anaerobic growth conditions, showed MICs of 100–200 mM of As(V), whereas the remaining isolates showed low levels of resistance to As(V), with MICs of 2 mM. Most bacterial isolates exhibited similar resistances to As(III) (MICs of 2.0–2.5 mM) as they did in aerobic growth conditions, except for the two *Pseudomonas* sp. strains (AR-2 and AR-3) that showed much higher tolerances to As(III) (MICs of 5 and 2.5 mM, respectively) than in aerobic growth conditions.

The abilities of the isolated bacteria to oxidize As(III) and reduce As(V) under aerobic and anaerobic growth conditions were further tested. All of the isolates except AR-11 were able to reduce As(V) to As(III) in both aerobic and anaerobic conditions (Table 3). The only As(III) oxidizer, strain AR-11, featured strictly aerobic growth and oxidized As(III) to As(V) in aerobic conditions (Table 3). The arsenic-transformation rates of strains AR-7 and AR-11 were further examined as they showed the most rapid As(V) reduction and As(III) oxidation rates, respectively among the isolates. To assess the As(V) reduction rate of AR-7, we used the well-studied arsenate-respiring strain, *Shewanella* sp. strain ANA-3 (Saltikov and Newman, 2003) as the reference strain and compared the As(V) reduction efficiencies of AR-7 and ANA-3 under the same culture conditions. In aerobic conditions, strain AR-7 rapidly reduced about 56% of As(V) to As(III) (0.14 mM) after 1.5 h of incubation. The nearly complete As(V) reduction to As(III) by AR-7 had occurred with 3 h of incubation (Fig. 2A). In contrast, *Shewanella* sp. strain ANA-3 had completely reduced As(V) to As(III) after 23 h of incubation (Fig. 2A). In anaerobic conditions, strain AR-7 exhibited a slower As(V) reduction rate than that in aerobic conditions. A 76% extent of As(V) reduction to As(III) by AR-7 was observed after 9 h of incubation. The complete As(V) reduction to As(III) by AR-7 was observed after 23 h of incubation (Fig. 2B). *Shewanella* sp. strain ANA-3 had rapidly reduced As(V) to As(III) (84%) after 3 h of incubation, while the nearly complete As(V) reduction to As(III) by ANA-3 was observed after 7.5 h of incubation (Fig. 2B). Fig. 2C shows As(III) oxidation to As(V) by strain AR-11. Strain AR-11 rapidly oxidized about 72% of As(III) (0.25 mM) to As(V) (0.18 mM) after 6 h of incubation. The complete As(III) oxidation to As(V) by AR-11 was observed after 12 h of incubation (Fig. 2C).

Additionally, the ability of strain AR-11 to transform arsenic at concentrations relevant to environmental groundwater was tested without the addition of any electron donors or acceptors. AR-11, the only As(III)-oxidizer, was selected, as arsenic in the environmental groundwater at the current sampling site mainly exists in the form of inorganic As(III) (780 μg L^{-1}, ~10.5 μM). Fig. 3 shows that AR-11 had rapidly oxidized 65% of As(III) to As(V) after only 30 min of incubation. The nearly complete As(III) oxidation to As(V) in the environmental groundwater by AR-11 was observed after 50 min of incubation.

3.3. Basic physiological characterization

We selected 2 facultative anaerobic As(V)-reducing bacteria (AR-4 and AR-5) and one aerobic As(III)-oxidizing bacterium (AR-11) for further basic physiological characterization. AR-4

Table 2
Bacterial strains genus identification and arsenic tolerance.

Bacterial isolate number	16S rRNA genus identification	MIC[a] (mM)			
		Aerobic		Anaerobic	
		As(III)	As(V)	As(III)	As(V)
AR-1	*Pseudomonas* sp.	2.5	150	2	2
AR-2	*Pseudomonas* sp.	2	100	5	100
AR-3	*Pseudomonas* sp.	2	200	2.5	2
AR-4	*Psychrobacter* sp.	2.5	200	2	2
AR-5	*Psychrobacter* sp.	5	100	2	2
AR-6	*Vibrio* sp.	2	200	2	2
AR-7	*Citrobacter* sp.	5	100	5	100
AR-8	*Enterobacter* sp.	5	200	5	200
AR-9	*Bacillus* sp.	5	200	2	100
AR-10	*Bacillus* sp.	5	200	5	200
AR-11	*Bosea* sp.	2	100	2	2

[a] MIC is defined as the lowest concentrations of As(III) or As(V) that completely inhibited bacterial growth on agar medium after 72 and 120 h of incubation for aerobic and anaerobic growth conditions, respectively.

Table 3
Arsenic transformation and arsenic marker gene detection of bacterial isolates.

Bacterial isolate number	Arsenic transformation		Arsenic marker gene			
	Aerobic	Anaerobic	*arrA*	*arsB*	*arsC*	*aoxB*
AR-1	Reduction[a]	Reduction	−[b]	+[c]	+	−
AR-2	Reduction	Reduction	−	−	−	−
AR-3	Reduction	Reduction	−	−	−	−
AR-4	Reduction	Reduction	−	−	+	−
AR-5	Reduction	Reduction	−	+	−	−
AR-6	Reduction	Reduction	−	−	+	−
AR-7	Reduction	Reduction	−	+	+	−
AR-8	Reduction	Reduction	−	+	+	−
AR-9	Reduction	Reduction	−	−	+	−
AR-10	Reduction	Reduction	−	−	+	−
AR-11	Oxidation[d]	N[e]	−	−	−	+

[a] As(V) reduction was analyzed after 120 h of incubation under aerobic or anaerobic condition.
[b] No positive PCR product generated.
[c] Presence of the marker gene and the PCR product was confirmed with sequencing.
[d] As(III) oxidation was analyzed after 72 h of incubation under aerobic condition.
[e] No growth under the conditions assessed.

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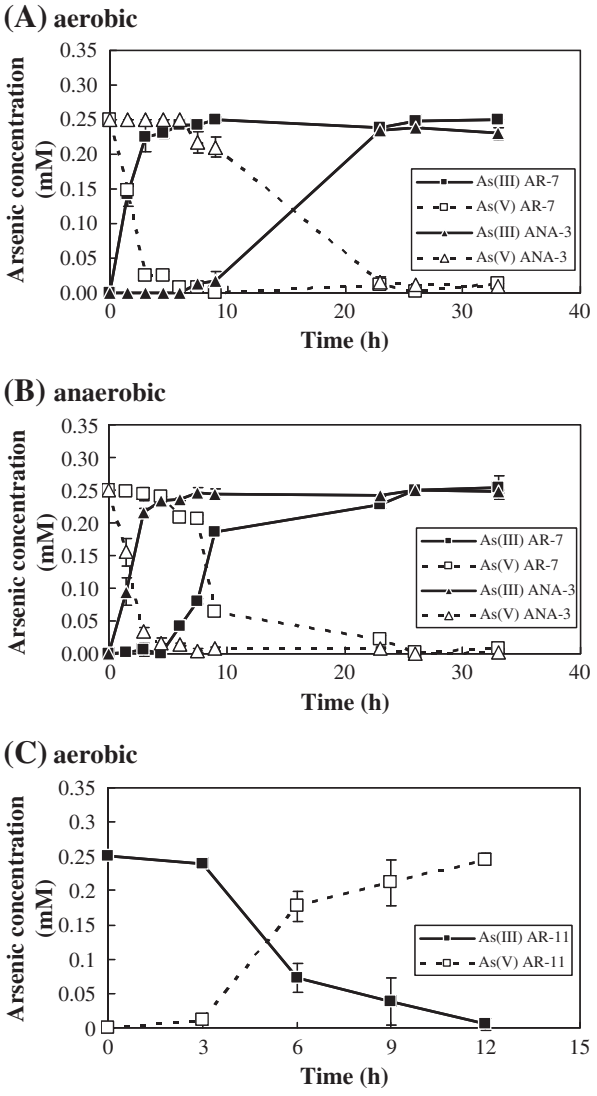

Fig. 2. Arsenic transformation by bacteria. As(V) reduction to As(III) by strain AR-7 and *Shewanella* sp. strain ANA-3 under aerobic (A) and anaerobic conditions (B). As(III) oxidation to As(V) by strain AR-11 under aerobic conditions (C). Each bacterial strain (with an optical density of 0.3 at 600 nm) was incubated in minimal salt medium containing 10 mM glucose and either 0.25 mM As(III) or As(V) at 25 °C. Samples were periodically withdrawn. Total concentrations of arsenic ([total As] = [As(III)] + [As(V)]) remained nearly constant during each experiment. Data are presented as the mean ± SD; symbols with no apparent error bars have error estimates too small to see or which are hidden by the symbol. Error bars represent the standard error of three replicate experiments.

and AR-5 were selected because they have so far not been described as catalyzing arsenic oxidation and reduction. Strain AR-11 was selected because it was the only As(III)-oxidizer among the isolates. We characterized the strains using the API ZYM system for the enzyme analysis and the API 50 CH system for the carbohydrate-utilization analysis. Detailed API ZYM and API 50 CH data are presented in Supplementary materials.

Strains AR-4 and AR-5 belong to the genus *Psychrobacter*. Both strains are gram-negative, rod-shaped, and non-motile. Strains AR-4 and AR-5 exhibited optimum growth at 30 °C, but growth occurred at 4–37 °C. Both strains are neutrophilic and grew best at pH 6.0–8.0, but not below pH 5.5 or above pH 9.0. AR-4 and AR-5 were relatively biochemically inert according to the commercial API ZYM test. AR-4 was only positive for naphthol-ASBI-phosphohydrolase and partially positive for acid phosphatase and α-glucosidase. AR-5 was positive for esterase lipase (C8), leucine arylamidase, and naphthol-ASBI-phosphohydrolase and partially positive for alkaline phosphatase, esterase (C4), and acid phosphatase. On the carbohydrate-utilization test, strain AR-4 was positive for glucose, fructose, mannose, mannitol, esculin, maltose, sucrose, and trehalose. AR-4 was also partially positive for the following carbohydrates: glycerol, ribose, D-xylose, *N*-acetyl-glucosamine, raffinose, starch, D-turanose, gluconate, and 5-keto-gluconate. Strain AR-5 utilized D-xylose, galatose, glucose, mannose, melibiose, gentiobiose, and D-fucose as carbon sources. AR-5 was also partially positive for L-arabinose, ribose, and fructose.

Strain AR-11 belongs to the genus *Bosea*. It is a gram-negative, rod-shaped, motile, and strictly aerobic bacterium. AR-11 grew at 20–37 °C, and it exhibited optimum growth at 37 °C. AR-11 grew best at pH 7.4–8.4, but not below pH 6.0 or above pH 9.0. AR-11 was positive for alkaline phosphatase, leucine arylamidase, acid phosphatase, naphthol-ASBI-phosphohydrolase, β-galactosidase, and α-glucosidase and partially positive for esterase (C4), cystine arylamidase, and β-glucuronidase. Strain AR-11 is an autotroph. It also grew well by utilizing the following carbohydrates: glycerol, D-arabinose, L-arabinose, ribose, D-xylose, galactose, glucose, fructose, mannose, rhamnose, mannitol, sorbitol, *N*-acetyl-glucosamine, lactose, melibiose, trehalose, gluconate, and 5-keto-gluconate.

3.4. Detection of arsenic marker genes

Several studies used genetic markers to study arsenic transformation mechanisms (Achour et al., 2007; Hamamura et al., 2009; Kulp et al., 2007; Malasarn et al., 2004; Rhine et al., 2007; Song et al., 2009; Sun et al., 2004). The presence of a genetic mechanism for arsenic resistance and arsenic transformation in each bacterial isolate was examined via PCR amplification of arsenic marker genes, and each PCR product was further sequenced to confirm detection of the target gene. The primers presented in Table 1 correspond to the *arsB*, *arsC*, *arrA*, and *aoxB* genes. Most isolated bacterial strains, except for strains AR-2 and AR-3, were found to be positive for at least one of the arsenic marker genes, yet differed in terms of the genes they carried (Table 3). For example, the *Pseudomonas* sp. strain AR-1 carried the *arsB* and *arsC* genes, whereas PCR failed to amplify arsenic marker genes for two other *Pseudomonas* sp. strains (AR-2 and AR-3) (Table 3).

3.5. Phylogenetic analysis

The genera of the bacterial strains were identified by analyzing their 16S rRNA genes (Table 2). Phylogenetic relationships were obtained by comparing the 16S rRNA sequences of the 11 bacterial isolates with sequences of members of previously reported arsenic-resistant, As(III)-oxidizing, and As(V)-reducing bacteria and closely related bacterial isolates. The 16S rRNA sequences of firmicutes were used as the outgroup (Fig. 4). Among the 11 isolated strains, eight were distributed

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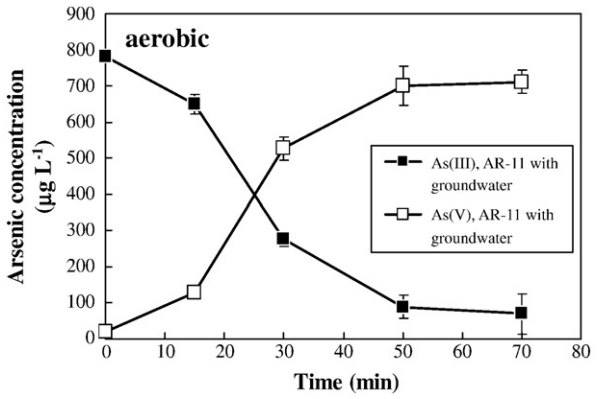

Fig. 3. Arsenite oxidation by strain AR-11 using environmental groundwater. AR-11 (with an optical density of 0.3 at 600 nm) was aerobically incubated at 25 °C in an environmental groundwater sample that was previously autoclaved until it was sterile without the addition of any electron donors or acceptors. The collected environmental groundwater contained 780 μg L^{-1} As(III). Samples were periodically withdrawn. Total concentrations of arsenic ([total As] = [As(III)] + [As(V)]) remained nearly constant during each experiment. Data are presented as the mean ± SD; symbols with no apparent error bars have error estimates too small to see or which are hidden by the symbol. Error bars represent the standard error of three replicate experiments.

into four classes of gamma-proteobacteria, including the families Pseudomonadaceae, Moraxellaceae, Enterobacteriaceae, and Vibrionaceae. In the clade of the Pseudomonadaceae, AR-1 was most closely related to *Pseudomonas aeruginosa* (100% sequence similarity), while AR-2 and AR-3 were most closely related to *Pseudomonas rhizosphaerae* (97% sequence similarity). AR-4 and AR-5 belonged to *Psychrobacter*, and shared 99% sequence similarity with the closest relative, *Psychrobacter glacicola*. AR-6, AR-7, and AR-8 were identified to be most closely related to *Vibrio fluvialis* (100% sequence similarity), *Citrobacter* sp. (99% sequence similarity), and *Enterobacter* sp. (99% sequence similarity), respectively. Two isolates, AR-9 and AR-10, were shown to be in the genus *Bacillus*, and shared 99% sequence similarity with the closest relative *Bacillus arseniciselenatis*. In contrast to the arsenate-reducing isolates dispersed in bacilli and gamma-proteobacteria, AR-11, the only isolate capable of arsenite oxidation, was identified to be in the cluster of alpha-proteobacteria. AR-11 shared 98% sequence similarity with *Bosea* sp.

4. Discussion

Microbial species that biotransform arsenic between oxidation states with differing environmental behaviors control the release of adsorbed arsenic by sediments into groundwater and may potentially be utilized for bioremediation. To date, most investigations of bacterial communities only focused on analyzing population shifts in incubation experiments using environmental samples from contaminated sediments in Southeast Asia. Although previous reports supported the biogeochemical basis for arsenic mobilization, such incubation experiments failed to identify the responsible *in situ* microbial populations (Islam et al., 2004; Rowland et al., 2009). In this study, we investigated the relevant As(III)-oxidizing and As(V)-reducing microbial community that exists in highly arsenic-contaminated groundwater that was collected from the vadose zone of the aquifer. Recently, Sutton et al. (2009) showed that the bacterial community identified in groundwater of shallow tube wells in Bangladesh showed similarities to those identified in As-contaminated sediments. This supports isolated microbes in the present study being representative of field sites.

Both aerobic and anaerobic conditions were evaluated because arsenic-contaminated groundwater in this study was collected from the vadose zone. We isolated 11 bacterial strains with different colony morphologies that grew in the presence of high concentrations of As(III) and As(V). For example, strain AR-8 grew in the presence of high concentrations of As(III) (up to 5 mM) and As(V) (up to 200 mM). This characteristic allows these bacteria to cope with the high arsenic concentrations in native groundwater. The isolated bacteria were grouped into the following genera: *Pseudomonas*, *Bacillus*, *Psychrobacter*, *Vibrio*, *Citrobacter*, *Enterobacter*, and *Bosea*. The gamma-proteobacteria were particularly well represented in this study with 7 of the 11 isolates corresponding to members of this group. This is not surprising, as gamma-proteobacteria have previously been found in arsenic-contaminated environments (Achour et al., 2007; Chang et al., 2008). *Pseudomonas* and *Bacillus* are broadly represented among arsenic-resistant bacterial strains isolated from other arsenic-contaminated sites (Anderson and Cook, 2004; Chang et al., 2008; Pepi et al., 2007). To the best of our knowledge, *Psychrobacter* was not previously reported in the literature as either an arsenic-resistant or arsenic-transforming genus. In a recent study, arsenic-resistant bacteria isolated from an estuary were identified as belonging to six major bacterial groups (Jackson et al., 2005). In addition, arsenic-resistant bacteria isolated from soils were identified as belonging to 22 major bacterial groups (Achour et al., 2007). Those previous results, together with the bacterial strains isolated in the present study, indicate that arsenic-resistant bacteria appear to be widely distributed in natural environments and are phylogenetically diverse.

All of the isolates in the present study were resistant to As(III) or As(V), and some of the bacteria exhibited high resistance to As(V). Microbial resistance to concentrations of As(V) exceeding 100 mM is considered very high (Jackson et al., 2005). Notably, under aerobic growth conditions, all of the bacterial isolates exhibited high resistance to As(V) with MICs exceeding 100 mM. Several factors, such as the method of determining the resistance and the medium composition, can affect arsenic bioavailability and toxicity, resulting in discrepancies in MIC values (Achour et al., 2007). Therefore, MICs in the present study were not directly compared to those reported by others.

Most isolated arsenic-resistant bacterial strains in the present study were found to be positive for at least one of the arsenic marker genes, yet differed with respect to the genes they carried (Table 3). For example, the *Psychrobacter* sp. strain AR-4 encoded the *arsC* gene, whereas the other *Psychrobacter* sp. strain, AR-5, contained the *arsB* gene. *arsB* and *arsC* are part of the same operon and thus are regulated together. The absence of *arsB* or *arsC* as observed in our isolates is probably indicative of variations in the gene that decreased the homology of our primer sets. The *Pseudomonas* sp. strain, AR-1, encoded both *arsB* and *arsC* genes, whereas the PCR failed to amplify any arsenic marker genes for the two *Pseudomonas* sp. strains AR-2 and AR-3 (Table 3). This is not surprising since several studies showed that no *ars* genes were

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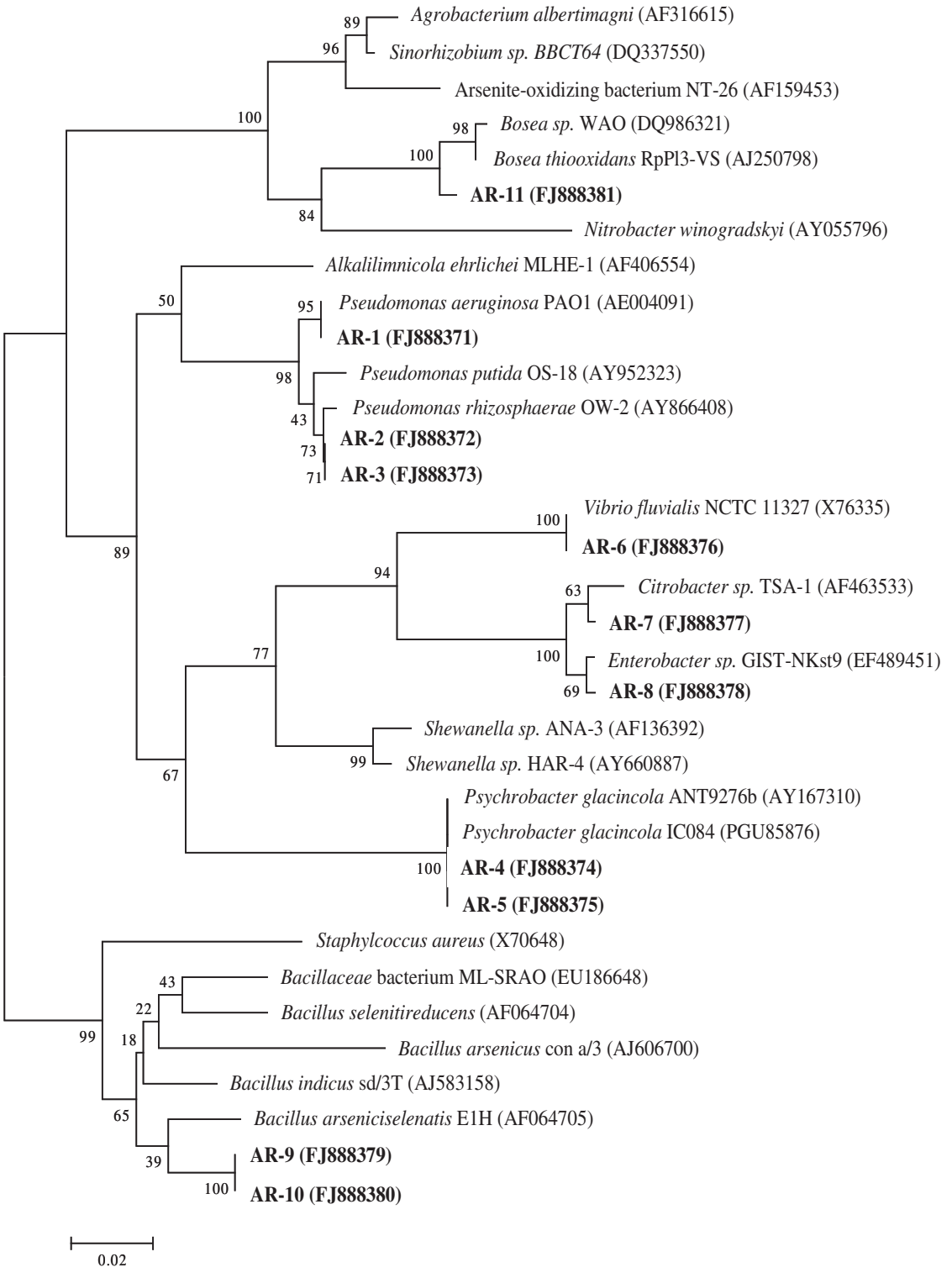

Fig. 4. 16S rRNA gene neighbor-joining tree showing the phylogenetic relationships of 11 arsenic-resistant isolates with previously reported arsenic-resistant, As(III)-oxidizing, and As(V)-reducing bacteria or closely related bacterial isolates from GenBank. The scale bar represents the number of substitutions per site within the 16S rRNA gene.

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found in *Pseudomonas* sp. (Cai et al., 1998; Chang et al., 2007; Saltikov and Newman, 2003; Saltikov et al., 2003), suggesting that the *ars* operon is not always the same, even though the strains were categorized into the same genera. Therefore, this type of diversity should be considered when *ars* systems are examined using PCR-based techniques. Furthermore, many factors such as the designed primers, thermal cycling conditions, and the composition of the buffers or agents in a PCR analysis can influence the amplification of *ars* genes and unknown chromosomal *ars* areas (Chang et al., 2007).

All of the isolates except AR-11 were able to reduce As(V) to As(III) in both aerobic and anaerobic conditions. This might be because the groundwater samples were collected from the vadose zone of the aquifer where the environment can vary between aerobic and anaerobic conditions. It is noteworthy to point out that As(V) reduction can be achieved via a process of anaerobic respiration using As(V) as a terminal electron acceptor, or via a detoxification mechanism using ArsC As(V)-reductase. This implies that isolates (AR-1 to AR-10) displaying As(V) reduction ability in this study contain either the anaerobic respiratory As(V) reductase gene or the *arsC* gene or both in their genome. Strain AR-7 reduced As(V) to As(III) faster than did *Shewanella* sp. strain ANA-3 in an aerobic condition under our experimental conditions, whereas *Shewanella* sp. strain ANA-3 exhibited a faster As(V) reduction rate than that of AR-7 in an anaerobic condition. ANA-3 was previously shown to reduce As(V) to As(III) in both aerobic and anaerobic conditions (Saltikov et al., 2003). However, studies showed that the *ars* detoxification system is not required for As(V) respiration by ANA-3 (Saltikov et al., 2003). Strain AR-11 was the only As(III)-oxidizer in this study. AR-11 is an autotroph, and it featured strictly aerobic growth and was able to rapidly oxidize As(III) to As(V) under aerobic conditions. Particularly, strain AR-11 exhibited a rapid As(III) oxidation rate at concentrations relevant to the environmental groundwater sample without the addition of any electron donors or acceptors. In addition, there is a good agreement of the presence of the *aoxB* marker gene in the genome of the AR-11 strain. Among our isolates, there was no clear relationship between resistance levels and the prevalence of arsenic marker genes.

5. Conclusions

The processes controlling the release of arsenic into groundwater systems were extensively studied over the past decade, but still remain a subject of intense debate. Various mobilization mechanisms were proposed, including oxidation of arsenic-rich pyrite (Das et al., 2004), reductive dissolution of iron oxyhydroxide phases (Harvey et al., 2002; Nickson et al., 2000), competition of solutes for sorption sites on iron oxides (Acharyya et al., 1999), and microbial reduction of sorbed As(V) to the potentially more-mobile As(III) under anoxic conditions (Oremland and Stolz, 2003, 2005). In this study, we provide the first look at the relevant microbial community that exists in an arsenic-contaminated aquifer that is historically linked to extreme arsenic toxicity in Taiwan. Our results indicate that a relatively diverse community of microorganisms capable of environmentally significant biotransformations of arsenic is naturally present in the aquifer. The identified bacteria appear to be well adapted to arsenic concentrations that significantly exceed those found in the aquifer. This information is important since effective *in situ* bioremediation technology significantly depends on the activity of indigenous microbial communities at contaminated sites. Furthermore, this work is the first report describing the As(V)-reducing activity by *Psychrobacter* which was not previously reported in the literature. Additionally, we report here that PCR methods failed to amplify any arsenic marker genes for two of the isolated *Pseudomonas* strains, despite the fact that both strains reduced As(V) in culture, suggesting that the *ars* operon may vary within strains of the same genera. This has wide-ranging implications for other localities indicating that the currently available primer sets are not actually suitable to detect the *ars* operon in all cases. Moreover, the rapid oxidization of As(III) to the less-toxic and more highly sorptive As(V) by strain AR-11 opens the way to its potential application in biological treatment of arsenic contamination at this and other arsenic-contaminated sites.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.jconhyd.2010.12.003.

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