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Iron Cycling Potentials of Arsenic Contaminated Groundwater in Bangladesh as Revealed by Enrichment Cultivation

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ABSTRACT

The activities of iron-oxidizing and reducing microorganisms impact the fate of arsenic in groundwater. Phylogenetic information cannot exclusively be used to infer the potential for iron oxidation or reduction in aquifers. Therefore, we complemented a previous cultivation-independent microbial community survey covering 22 arsenic contaminated drinking water wells in Bangladesh, with the characterization of enrichments of microaerophilic iron oxidizers and anaerobic iron reducers, conducted on the same water samples. All investigated samples revealed a potential for microbial iron oxidation and reduction. Microbial communities were phylogenetically diverse within and between enrichments as was also observed in the previous cultivation-independent analysis of the water samples from which these enrichments were derived. Enrichment uncovered a larger diversity in iron-cycling microorganisms than previously indicated. The iron-reducing enrichments revealed the presence of several 16S ribosomal RNA (16S rRNA) gene sequences most closely related to *Acetobacterium*, *Clostridium*, *Bacillus*, *Rhizobiales, Desulfovibrio*, *Bacteroides*, and Spirochaetes, in addition to well-known dissimilatory iron-reducing *Geobacter* and *Geothrix* species. Although a large diversity of *Geobacteraceae* was observed, they comprised only a small part of the iron-reducing consortia. Iron-oxidizing gradient tube enrichments were dominated by *Comamonadaceae* and *Rhodocyclaceae* instead of *Gallionellaceae*. Forty-five percent of these enrichments also revealed the presence of the gene encoding arsenite oxidase, which converts arsenite to less toxic and less mobile arsenate. Their potential for ferric (oxyhydr)oxides precipitation and arsenic immobilization makes these iron-oxidizing enrichments of interest for rational bioaugmentation of arsenite contaminated groundwater.

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KEYWORDS
Fe(II) oxidation; Fe(III) reduction; drinking water wells; arsenic contamination; *aioA*

Introduction

Iron exists in groundwater systems predominantly in ferrous [Fe(II)] and ferric [Fe(III)] states (Chapelle 2001). At or above circumneutral pH, ferric iron primarily occurs as insoluble iron (oxyhydr)oxide minerals. In contrast, below pH 4.0 or under microaerophilic to anaerobic conditions, ferrous iron is relatively soluble in water and is mobile (Weber et al. 2006a). Microorganisms play a role in the cycling between the two redox states. Oxidation of organic carbon by heterotrophic iron-reducing bacteria is the dominant mechanism for ferric iron reduction in anaerobic groundwater systems (Lovley and Anderson 2000; Weber et al. 2006b). In contrast, chemolithotrophic microorganisms can obtain energy through the oxidation of ferrous iron in aerobic acidic environments (Clarke et al. 1997; Kozubal et al. 2008), at neutral pH under microaerophilic conditions (Emerson and Weiss 2004; Hallberg and Ferris 2004) or under nitrate reducing conditions (Weber et al. 2006a).

Iron can create, directly or indirectly, a number of nuisances in the extraction and human use of groundwater. Its oxidation can cause significant clogging problems in drinking water extraction (Ghiorse 1984; Emerson and De Vet 2015). Although iron is not considered to cause health problems in humans, its presence in potable water is rather unpleasant due to its rusty taste, the bad odors it spreads, and its tendency to stain clothing red. The emergence of red water in drinking water distribution systems might be caused by the activity of microaerophilic iron oxidizers, leading to extensive precipitation of iron (oxyhydr)oxides (Li et al. 2010).

Furthermore, the microbial mediated redox cycling of iron significantly influences the mobilization of toxic elements such as arsenic (Zobrist et al. 2000; Islam et al. 2004; Oremland and Stolz 2005). Several studies have documented a moderate to strong correlation between ferrous iron and arsenic in groundwater (e.g., Nickson et al. 2000; McArthur et al. 2001; Höhn et

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†Deceased 25 September 2015.

*The original corresponding author, Dr. Wilfred F. M. Röling, passed away 25 September 2015. He has been in charge of the entire manuscript, except for two final textual changes. The authors would like to dedicate this paper to the fond memory of Dr. Wilfred Röling, who did so much for systems ecology and microbiology.

Supplemental materials for this article can be accessed on the publisher's website.

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Table 1. Enrichment of iron cycling microorganisms initiated from 22 drinking water wells in Bangladesh (indicated by sample code or ID, name of the village in which the well is located, district, well depth, and physicochemical parameters).

Location			Physicochemical parameters					Iron-oxidizing enrichments				Iron reducing enrichments			
Sample ID	Name of the village	District	Depth (meter)	pH	As (μg/l)	Fe (mg/l)	NO_3 (mg/l)	Dilution factor	Bacteria 16S rRNA	*Gallionellaceae* 16S rRNA	Arsenite oxidase	Dilution factor	Bacteria 16S rRNA	*Desulfuromonadales* 16S rRNA	Arsenate reductase
A1	Assasuni sadar	Satkhira	13.7	6.78	118.0	14.30	24.46	3	+	−	−	3	+	+	−
N1	Nagda		146.3	8.00	4.5	0.22	0.05	3	+	−	+	3	+	+	+
NA1	Nawapara1		22.9	6.94	6.5	9.03	0.08	3	+	−	−	2	+	+	−
T1	Tarali		48.8	6.75	248.0	3.41	0.06	3	+	−	−	3	+	+	−
NA2	Nawapara2		48.8	6.85	262.0	3.08	2.97	3	+	−	−	3	+	+	+
K1	Kaliganj sadar		29	6.81	0.4	5.43	25.26	3	+	−	−	3	+	+	−
Vu	Vurulia		27.4	8.02	235.0	6.97	0.40	3	+	−	−	ND	ND	ND	ND
Mu	Munshiganj		80.8	6.65	0.02	0.27	0.19	2	+	−	−	ND	ND	ND	ND
Bp	Boropukut		62.5	7.02	2.6	0.22	0.05	2	+	−	−	ND	ND	ND	ND
Hn	Henchi		54.9	6.56	77.6	6.53	0.05	3	+	+	+	ND	ND	ND	ND
Jn	Jaynagar		13.7	6.63	203.0	8.06	0.05	3	+	−	+	ND	ND	ND	ND
Gp	Gopalpur		51.8	7.79	623.0	16.30	0.07	3	+	−	+	ND	ND	ND	ND
Ts	Tirerhat	Jessore	176.8	6.71	463.0	11.10	0.53	3	+	+	−	ND	ND	ND	ND
Td	Tirerhat-deep		207.3	6.35	103.0	1.91	0.45	3	+	−	+	ND	ND	ND	ND
M1d	Magura-deep		25.7	6.57	78.6	1.74	0.11	3	+	−	−	ND	ND	ND	ND
M2s	Magura		24.1	6.08	303.0	7.48	0.14	3	+	−	−	ND	ND	ND	ND
Uz	Uzzalpur		36.6	6.19	132.0	7.72	0.11	3	+	−	+	ND	ND	ND	ND
Sm	Samta		21.3	6.25	162.0	7.60	1.04	3	+	−	+	ND	ND	ND	ND
Mn-40.1	Payob	Comilla	14.3	6.61	208.5	5.34	20.58	3	+	−	+	1	+	+	+
Mn-40.2	Payob		21.3	6.3	81.2	8.09	14.67	3	+	−	−	1	+	+	+
Mn-40.3	Payob		22.9	6.14	78.8	7.09	14.24	2	+	−	+	1	+	+	−
DK-8	Daudkandi		24.4	6.1	237.0	4.76	0.06	3	+	−	+	ND	ND	ND	ND
							Total	100%	100%	9%	45%	100%	100%	100%	44%

Microaerophilic iron oxidizers and anaerobic iron reducers were investigated. The column "dilution factor" presents the highest serial tenfold dilution at which still growth was observed. Subsequent columns indicate the results of molecular analysis of specific 16S rRNA genes or functional genes indicative of potential for arsenic redox transformations. +: detected, −: not detected, ND: not determined.

al. 2006). Arsenic is highly toxic to humans (Lloyd and Oremland 2006). Natural arsenic contamination in drinking water is a major concern for public health in Bangladesh and other countries in South and Southeast Asia, where people drink arsenic contaminated groundwater (Yu et al. 2003). These iron and arsenic are released into the groundwater due to the reductive dissolution of iron minerals containing arsenic (Islam et al. 2004). In contrast, aqueous arsenic contamination can be removed through biological oxidation of ferrous iron, as the precipitating ferric (oxyhydr)oxides bind arsenic (Katsoyiannis and Zouboulis 2004).

Thus, the activity of iron-reducing and oxidizing microorganisms indirectly affects arsenic concentrations in groundwater, in addition to the activity of specific microorganisms directly involved in the biogeochemical cycling of arsenic: arsenate-reducing bacteria releasing arsenite [As(III)] and arsenite-oxidizing bacteria producing arsenate [As(V)], which is less toxic, less mobile, and binds better to ferric (oxyhydr)oxides than arsenite (Cavalca et al. 2013). Knowledge on the microbial ecology of the iron cycle in relation to the generation and remediation of arsenic contaminated drinking water in South and Southeast Asia is still limited, in particular with respect to iron oxidation potential. Previously, we sampled 24 drinking water wells from four districts in Bangladesh and conducted a cultivation-independent 16S rRNA gene-based survey to obtain insight into the occurrence of microorganisms with potential for iron-cycling (Hassan et al. 2015). However, 16S rRNA gene-based molecular techniques do not necessarily inform on the physiological traits of the identified microorganisms. The capability to reduce iron is spread over many bacterial and archaeal genera, and within several of these genera some members do reduce iron while others do not (Lovley et al. 2004). Furthermore, it has become evident that the capability to oxidize iron is present in more genera than previously thought (Hedrich et al. 2011).

Therefore, to understand the role of iron-cycling in arsenic contaminated groundwater, it appeared essential to follow up our previous cultivation-independent work on iron-cycling with cultivation studies. Accordingly, this study aims to survey the abundance, distribution, and diversity of cultivatable iron-oxidizing and iron-reducing microorganisms in arsenic contaminated drinking water wells in Bangladesh. We hypothesized that a diverse range of cultivatable iron-cycling microorganisms is present in arsenic contaminated groundwaters in Bangladesh. We also hypothesized that enrichment would reveal iron-cycling microorganisms that had not been identified on the basis of our previous cultivation-independent analysis (Hassan et al. 2015) of the very same 22 samples investigated here. Furthermore, we investigated metabolic flexibility by determining the presence of arsenite oxidase (*aioA*) and arsenate reductase (*arrA*) genes, which would indicate the potential of the enrichments to oxidize arsenite or reduce arsenate, respectively.

Materials and methods

Field sampling

Between August 2011 and March 2012, a total of 22 groundwater samples were collected from shallow and deep tube wells from the Satkhira, Jessore, and Comilla districts in Bangladesh (Table 1) (Hassan et al. 2015). Anaerobic groundwater samples were collected in sterile serum glass bottles by letting the bottles overflow, after three volumes of standing water in each tube well had been removed by hand pumping. Bottles with groundwater samples for culturing were capped with as little headspace as possible and transferred to the laboratory, where they were stored for less than 24 h at 4°C. Details on the hydrochemistry of these samples were reported in our previous study (Hassan et al. 2015), with key features indicated in Table 1. All samples were anaerobic, with pH values between 6.1 and 8.0.

Enrichment of microaerophilic iron-oxidizing microorganisms

A gradient tube cultivation approach with opposing gradients of oxygen and ferrous iron was employed to enrich and maintain microaerophilic iron oxidizers (Emerson and Floyd 2005). Modified Wolfe's Mineral Medium (MWMM) consisted of the following ingredients (in g l^{-1} distilled water): NH_4Cl, 1.0; $MgSO_4.7H_2O$, 0.2; $CaCl_2.2H_2O$, 0.1; K_2HPO_4, 0.05; and $NaHCO_3$, 4.2. The system consisted of two layers of agarose in 16-mm screw cap glass tubes (Wang et al. 2009). The bottom layer contained 1.0 ml of FeS or $FeCO_3$ solution and MWMM at a ratio of 1:1, amended with 1% (wt/vol) agarose. $FeCO_3$ and FeS stock solutions were prepared according to Hallbeck et al. (1993) and Emerson and Floyd (2005), respectively. The top layer consisted of 5.0 ml of MWMM supplemented with 5 μl of vitamin solution (medium 141, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH [DSMZ]) and trace element solution each (medium 141, DSMZ), stabilized with 0.15% (wt/vol) agarose. Before autoclaving, the solutions for the upper layer were mixed with 0.5 ml of 50 mM $NaHCO_3$ and flushed with CO_2 for 2–3 min to attain a final pH of 6.4. The gradient tubes were inoculated ~24 h after preparation to facilitate the diffusion of ferrous iron into the top layer where bacterial growth occurs. Tenfold serial dilutions (up to 10^{-3}) of groundwater samples in sterile 0.85% NaCl solution were inoculated into gradient tubes. The tubes were inoculated just above the iron plug by pulling a micropipette vertically through the medium while the inoculum (10 μl) was added slowly from the micropipette. Abiotic controls were prepared without bacteria (an uninoculated control and a control inoculated with 10 μl sterile 0.85% NaCl) to distinguish between abiotic and biotic iron oxidation. Tubes were incubated in the dark at 28°C for 2–3 weeks. Microbial growth in the gradient tube was identified by the formation of a discrete brownish iron oxide band, after visual comparison to the more diffused bands that typically develop due to chemical oxidation of ferrous iron in abiotic control tubes (Supplementary Figure S1a). Three to four serial transfers were performed to obtain the dominant iron oxidizers; a sterile inoculating needle was used to extract cell material from the discrete brownish band and to transfer this material into a fresh tube. A Pasteur pipette was used to obtain bacterial cell material (~0.5–1.0 ml) for molecular analysis. Cell material was centrifuged and the pellets were stored at−20°C until DNA extraction.

Enrichment of anaerobic iron-reducing microorganisms

Strictly anaerobic techniques were used to enrich iron-reducing microorganisms. The anoxic basal medium was

based on Lin et al. (2007) and contained (in g l^{-1} distilled water) KCl, 0.1; NH_4Cl, 1.5; NaH_2PO_4, 0.6; $NaHCO_3$, 2.5; $Na_2WO_4.2H_2O$, 0.00025; 10 ml of trace element solution; and 10 ml of vitamin solution (medium 141, DSMZ), amended with 2.0 mM Na lactate and 3.0 mM Na acetate. A 100 mM stock solution of Fe(III)–nitrilotriacetic acid (NTA) was prepared by dissolving 1.64 g of $NaHCO_3$, 0.256 g of trisodium NTA, and 0.27 g of $FeCl_3.6H_2O$ in water to a final volume of 100 ml. The solution was made anoxic by purging with a O_2-free mix of N_2 and CO_2 (80/20%), filter sterilized, and stored in an anaerobic, sterile serum bottle (Liu et al. 2002). We used Fe(III)–NTA (final conc. 5.0 mM) as an electron acceptor, resazurin dye (0.0005 g l^{-1}) as an indicator for the presence of oxygen, and 0.1 mM cysteine as an oxygen scavenger. Anaerobic medium was prepared in serum vials sealed with butyl rubber septa and crimped with aluminum caps. The basal medium was flushed with a mix of N_2 and CO_2 (80/20%). Iron-reducing enrichments were performed on nine groundwater samples (Table 1). One milliliter of sample was inoculated into an anaerobic serum vial containing 9 ml of medium. These cultures were tenfold serial diluted up to 10^{-3} in the same medium and incubated in the dark at 28°C for 2–3 weeks. Positive iron reduction (medium turning colorless) was inferred by comparison with an uninoculated control (no color changes) (Supplementary Figure S1b). The highest dilution revealing iron reduction was used to inoculate fresh medium (2% vol/vol) and incubated again. This procedure was repeated three to four times.

For molecular analysis, iron-reducing cultures were vacuum-filtered over 45-mm-diameter, 0.2-μm pore size nitrocellulose membrane filters (Millipore, Billerica, MA, USA) and frozen at −20°C until DNA isolation.

DNA extraction

DNA was extracted using the soil DNA extraction kit (Mo BIO Laboratories Inc., Carlsbad, CA, USA) according to manufacturer's instructions. DNA was stored at −20°C.

16S rRNA gene-based profiling of bacterial, Gallionellaceae and Desulfuromonadales communities

Partial 16S rRNA gene sequences of bacteria, iron-oxidizing *Gallionellaceae*, and iron-reducing *Desulfuromonadales*, were amplified for denaturing gradient gel electrophoresis (DGGE) analysis, using the same primers and amplification conditions as in our previous study (Hassan et al. 2015; Supplementary Table S1). For bacteria, primer set 357F-GC clamp and 907r was used. The 16S rRNA gene fragments of *Gallionellaceae* were obtained with a nested PCR approach (Wang et al. 2009) using primer set 122F and 998R in the first round of amplification, followed by primer set 357F-GC and 907r in the second round, after diluting (1:100) the first round PCR products. A *Desulfuromonadales*-specific primer set (8f and 825r) was employed to amplify a 0.8-kb 16S rRNA gene fragment (Snoeyenbos-West et al. 2000). The order *Desulfuromonadales* comprises the iron-reducing families *Geobacteraceae* and *Desulfuromonadaceae* (Röling 2014). However, when Snoeyenbos-West et al. (2000) developed their primers, members of current *Desulfuromonadaceae* were still included in the family *Geobacteraceae*. PCR products were then diluted (1:100) and used for the second round of amplification using bacteria specific primers 357F-GC and 518r. Each PCR reaction was carried out using 25 μl (total volume) of mixture containing 12 μl of GoTaq (Promega, Madison, WI, USA) ready Master Mix, 1 μl of each primer (0.4 μM final concentration), 8 μl of nuclease free water (Promega), and 3 μl of undiluted DNA as template.

DGGE was carried out using a Dcode^{TM} Universal Mutation Detection System (Bio Rad Laboratories, CA, USA). PCR product was loaded onto a 1-mm-thick 8% (wt/vol) polyacrylamide (ratio of acrylamide to bisacrylamide, 37.5:1) gel containing a linear gradient of 30–55% of urea–formamide. The running conditions were 200 V at a constant temperature of 60°C in 1 × TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM Na-Ethylenediaminetetraacetic acid (EDTA), pH 8.0) for 4 h. The gels were stained in 1 × (Tris base, acetic acid and EDTA) (TAE) buffer containing 1 μg/ml of ethidium bromide and visualized using a UV transilluminator. To aid in the normalization of and comparison between gels, a DGGE marker (M12) with 12 bands at various positions was added to the external lanes of the gels, as well as to lanes in between every four samples. All gels to fingerprint a particular group of bacteria were run on the same day. The average between-gel similarity of the marker was 92%, with 3% standard deviation.

Bands were excised using sterile wide mouth blunt tips. Excised DNA bands were suspended in 1 × TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5) and stored overnight at 4°C. One microliter of suspension was used as a template in the aforementioned PCR, using primers without GC clamp. Products were checked on 1.5% agarose gels and sequenced (Macrogen, Amstelveen, The Netherlands).

Analysis of potential for arsenic metabolism in enrichments

A degenerate oligonucleotide primer set was used to amplify the gene encoding arsenite oxidase, *aioA* (Supplementary Table S1). Partial arsenate respiratory reductase (*arrA*) genes were amplified using primers ArrAfwd and ArrArev (Supplementary Table S1). Amplification often yielded faint *arrA* products, consisting of multiple bands. Bands with the expected size (∼160–200 bp) were cut from 2% low melting point agarose gels, reamplified, and rechecked by agarose gel electrophoresis. *aioA* and *arrA* products were subjected to sequencing (Macrogen).

Phylogenetic analysis

Sequences were aligned with ClustalW using default settings and were manually edited. Phylogenetic analyses were performed with MEGA 4 (Tamura et al. 2007). Nucleotide distance and amino acid Poisson correction analyses were performed through maximum composite likelihood computation and trees were constructed using the neighbor-joining method with a bootstrap value of 1000 replicates. Gene sequences have been deposited in GenBank under accession numbers: KR095355 to

KR095423 (bacterial 16S rRNA genes), KR095424 to KR095433 (*aioA*), and in the DNA Data Bank of Japan (DDBJ) database under accession numbers: LC043403 to LC043406 (*arrA*) and LC043407 to LC043433 (*Desulfuromonadales*-like 16S rRNA gene sequences).

Statistical analysis

Quantitative analysis of DGGE profiles was performed with GelCompar II (Applied Maths, Belgium) (van Verseveld and Röling 2004). Similarities between profiles were calculated using the Pearson correlation coefficient and visualized by the unweighted paired group clustering method with arithmetic means (UPGMA). The observed clusters of enrichments were related to hydrochemical characteristics of the groundwater from which these enrichments were derived, using nonparametric analysis of variance (Kruskal–Wallis) as described in our previous study (Hassan et al. 2015).

Results

Characterization of microaerophilic iron-oxidizing enrichments

All microaerophilic iron-oxidizing enrichments initiated with groundwater samples from 22 drinking water wells revealed iron oxidation within 7 days of incubation, most often at the highest dilution (10^{-3}) tested (Table 1, Supplementary Figure S1a). DGGE analysis after four serial transfers of these enrichments revealed that fingerprints were generally dominated by a few brightly stained bands and several less intense bands (Figure 1a: one to four bright bands per profile). Considerable variation between community profiles was observed: the fingerprints grouped into six clusters, at a 60% cutoff value (Figure 1a). No significant correlation was detected between these clusters and the hydrochemical characteristics of the groundwater from which the enrichments were derived (all $p > 0.05$; Kruskal–Wallis).

To gain insight into the identities of the major bacterial populations in the iron-oxidizing enrichments, 41 prominent DGGE bands were sequenced (see Figure 1a for banding positions and Figure 1b for phylogeny). The *Betaproteobacteria* constituted the dominant group (78% of the sequenced bands) followed by *Alphaproteobacteria* (15%) and *Gammaproteobacteria* (5%). *Acidobacteria* (*Geothrix fermentans*) contributed the remaining 2%. *Betaproteobacteria* sequences were most closely related to a variety of genera in especially the families *Comamonadaceae* and *Rhodocyclaceae*. Within the *Comamonadaceae* family, *Hydrogenophaga sp.* (11 bands; 27% of total), *Curvibacter sp.* (two bands; 5% of total), and *Acidovorax sp.* (two bands; 5% of total) were identified. *Hydrogenophaga* related bands were frequently detected in profiles belonging to clusters 1, 4, and 6 (Figure 1). Within the *Rhodocyclaceae* family, *Dechloromonas sp.* (four bands; 10% of total) and *Azonexus* (one band; 2% of total) were observed. *Dechloromonas* appeared to be confined to cluster 4, with bands labeled 8 in enrichment NA2, 10 in Mn-40.1, 29 in Ts, and 40 in Sm (Figure 1a). Furthermore, two bands relating to denitrifying iron-oxidizing bacteria were detected (5% of total; bands numbered 37 in sample Uz and 41 in Sm), while 14 genera of *Betaproteobacteria* were observed once, including a sequence with 97% similarity to the heterotrophic iron oxidizer *Leptothrix* (band 39 in Gp). Within

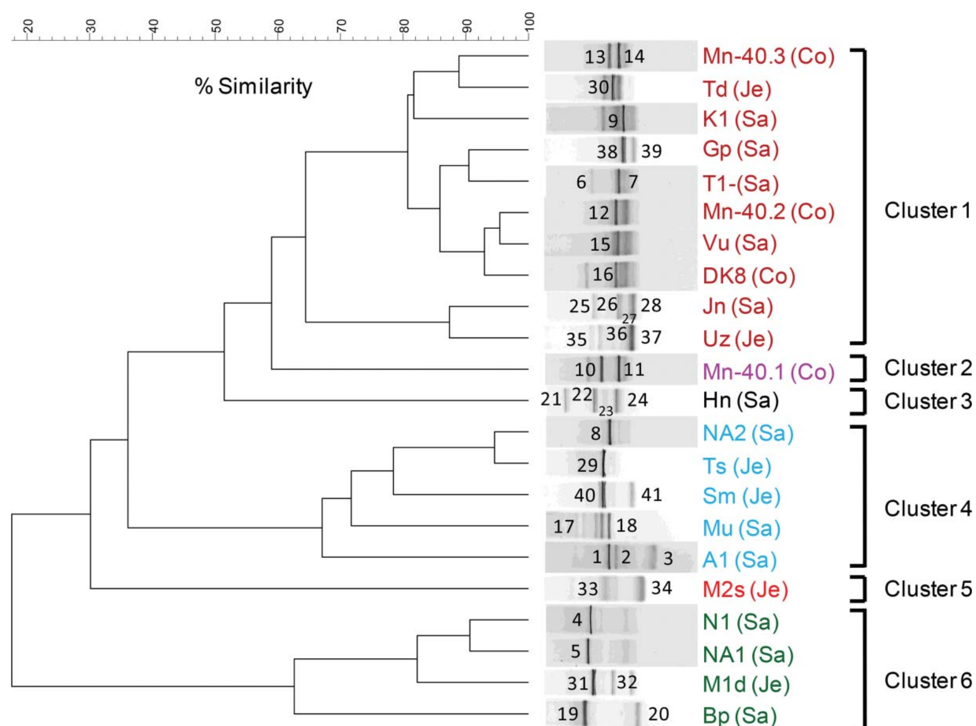

Figure 1. (a) UPGMA cluster analysis of bacterial 16S rRNA gene-based DGGE profiles (30–55% denaturant gradient) of 22 microaerophilic iron-oxidizing enrichments, using Pearson correlation analysis as a measure of similarity. The enrichment ID refers to the location of the drinking water well (see Table 1). Enrichments were assigned to clusters on the basis of >60% similarity. Names of enrichments belonging to a particular cluster received the same color, and this color is used in Figure 1b to indicate the sequences of their excised bands. Numbers refer to the position of excised bands.

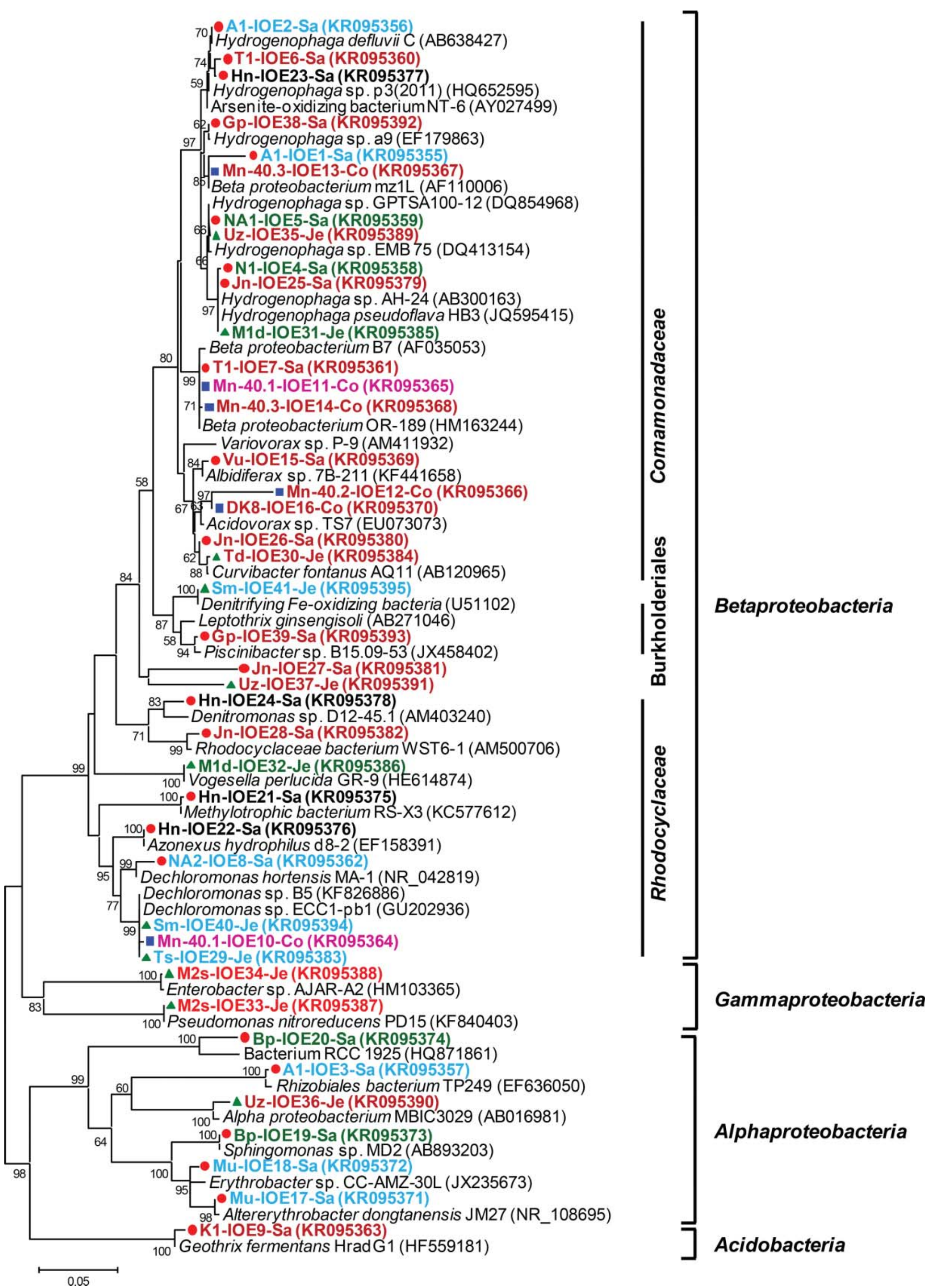

Figure 1. (b) Phylogenetic analysis of 16S rRNA gene sequences (525 unambiguously aligned nucleic acid positions) retrieved from the excised DGGE bands. Sequences are indicated by enrichment ID and the number of the excised band, as shown in (a). The three districts from which enrichments were obtained are indicated by distinct symbols: closed red circles, Satkhira; green triangles, Jessore; and blue squares, Comilla. The tree was constructed with the neighbor-joining method and bootstrap values (1000 replications) are indicated at the interior branches. The scale bar represents 5% sequence divergence.

the *Alphaproteobacteria*, sequences were most closely related to six different genera, corresponding to one band each (Figure 1). A *Gallionellaceae*-specific PCR revealed that these microaerophilic, chemolithotrophic iron oxidizers were only detectable in two enrichments (Table 1; Hn and Ts).

All iron-oxidizing enrichments were tested for the presence of the arsenite oxidase gene (*aioA*). Forty-five percent (10 out of 22 enrichments) was positive (Table 1). Amino acid sequences were all most closely related to the AioA sequences of known arsenite-oxidizing bacteria (Figure 2; 86–99% amino acid similarity). Among them, AioA sequences most closely related to those encountered in *Hydrogenophaga* and *Acidovorax* species accounted for 50% of the total. These sequences were found in enrichments initiated with Hn, Jn, Sm, Mn-40.3, and DK8 groundwaters, and also the DGGE fingerprints of these enrichments revealed dominant bands with 16S rRNA gene sequences most closely related to *Hydrogenophaga* and *Acidovorax*, with the exception of Sm (bands labeled 25 in Jn, 13 in Mn-40.3, 23 in Hn, and 16 in DK8 in Figure 1a). We also detected AioA sequences most closely related to AioA found in *Paracoccus*, *Sinorhizobium*, *Bradyrhizobium*, and *Ancylobacter* (Figure 2). However, none of the 16S rRNA gene sequences of excised DGGE bands was closely related to any of these genera. The AioA sequences retrieved clustered well with

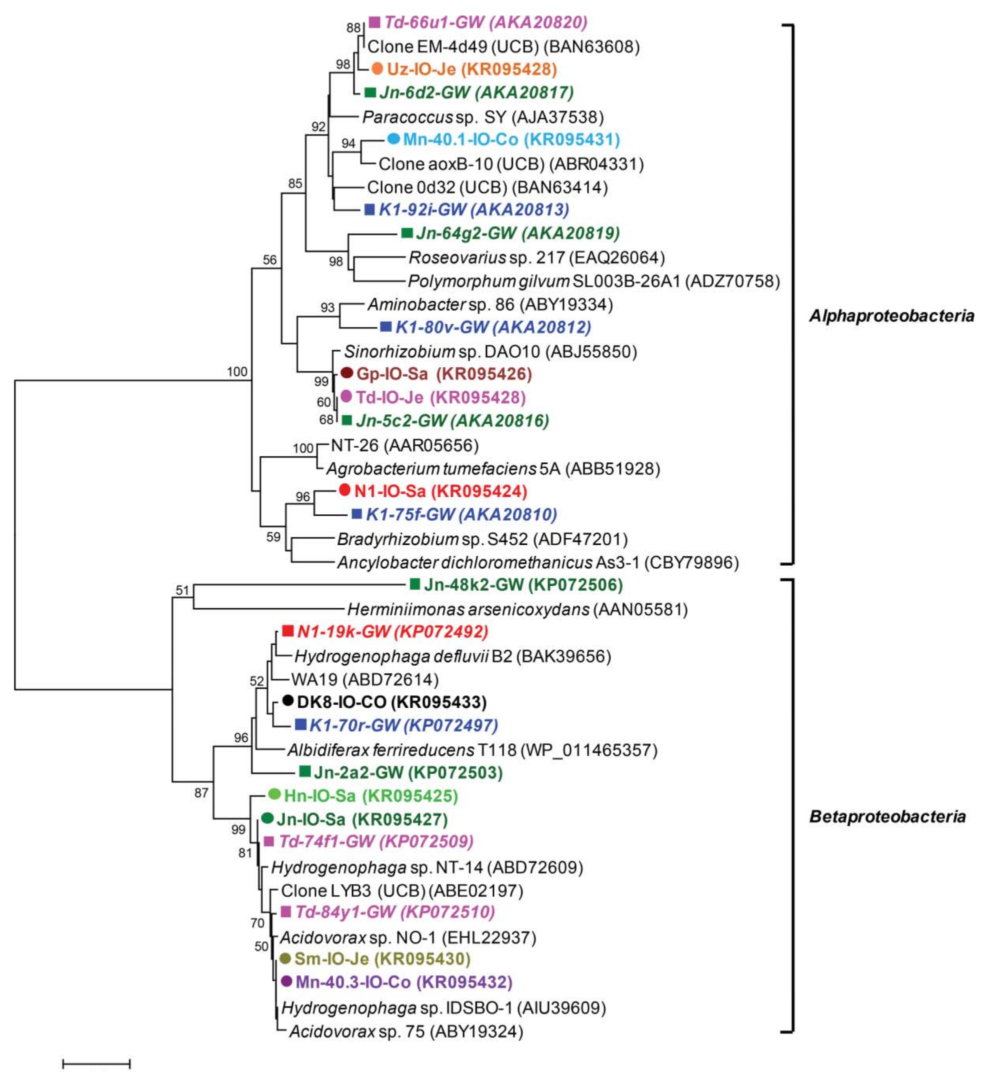

Figure 2. Unrooted neighbor-joining tree of amino acid sequences (162 unambiguously aligned positions) of the bacterial arsenite oxidase gene retrieved from iron-oxidizing enrichments. Bootstrap values (1000 replications) are indicated at the interior branches (bar = 0.1 substitutions/sequence position). The colored circles indicate the enrichments, with different colors referring to the various drinking water wells from which the enrichments were initiated. IDs in italics with colored squares indicate sequences derived directly from groundwater samples, without intermediate culturing (Hassan et al. 2015).

AioA sequences detected in our previous cultivation-independent analysis (Hassan et al. 2015) (Figure 2).

Characterization of iron-reducing enrichments

A total of nine groundwater samples were used to initiate iron-reducing enrichments. Iron was reduced in all enrichments, as indicated by the formation of ferrous iron after 3 weeks of incubation (Table 1). In five out of nine cases, iron reduction was observed at the highest dilution tested, that is, 10^{-3}. Enrichments maintained their iron reduction capability during three serial transfers (Supplementary Figure S1b). Subsequent DGGE analysis revealed several dominant bands for each enrichment (two to five bands per profile), but considerable variation between the enrichments (18 different banding positions in nine profiles) was observed (Figure 3a).

A total of 28 predominant bands were excised for sequence analysis. Phylogenetic diversity was high, besides members of several proteobacterial classes (*Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, and Deltaproteobacteria), Acidobacteria, Bacteroidetes, Firmicutes, and Spirochaetes* were observed) (Figure 3b). Many bands (46%, 13 bands observed in seven out of nine enrichments) were most closely related to various Firmicutes genera, mostly *Acetobacterium* (21%, six bands in three enrichments—bands numbered 12 in T1; 14, 15 in NA2; and 19, 20, and 21 in K1 in Figure 3a) and *Clostridium* (18%, five bands in four enrichments; bands labeled 5 and 6 in N1, 24 in Mn-40.2, 17 in K1, and 2 in A1). *Betaproteobacteria* and *Deltaproteobacteria* each contributed 11% of the sequenced bands (three bands each; Figure 3b). The *Betaproteobacteria* sequences (Mn-40.3, NA1, and A1) belonged to various genera: *Burkholderia* and two genera in the *Rhodocyclaceae*, *Dechloromonas* and *Azospira* (Figure 3b). All *Deltaproteobacteria* sequences were most closely related to sulfate reducing *Desulfovibrio* (Figure 3b: bands labeled 4 in A1, 9 in NA1, and 23 in Mn-40.1; 90–99% nucleotide similarity). Other phyla contributed 4–7% of the sequenced bands, including an *Acidobacterium* sequence most closely related to the known iron reducer *Geothrix fermentans* (band 8 in NA1 in Figure 3; 79% similarity).

DGGE analysis after a PCR specific for iron-reducing *Geobacteraceae* and *Desulfuromonadaceae* indicated their presence and diversity within enrichments (Figure 4a: one to eight bands per profile), as well as variation between the enrichments (20 different banding positions in nine profiles). As the employed primer set is not 100% specific for *Desulfuromonadales* (Snoeyenbos-West et al. 2000, Lin et al. 2005), a total of 35 DGGE bands were cut out and sequenced. Phylogenetic analysis revealed that most sequences were closely related to *Geobacteraceae* (77%, 27 bands in nine enrichments; Figure 4b). The sequencing confirmed the diversity of the *Geobacteraceae* within and between enrichments.

Testing for the presence of the arsenate reductase gene (*arrA*) in the iron-reducing enrichments revealed that some 44% (four out of nine; Table 1) was positive and contained *arrA* genes most closely related (80–99% nucleotide similarity) to those of *Sulfurospirillum*, *Geobacter*, or uncultured bacteria (Table 2). We did not observe any obvious correspondence between identities based on *arrA* sequences and identities based on bacterial 16S rRNA sequences retrieved from the same iron-reducing enrichments.

Discussion

Microbial diversity in iron-oxidizing enrichments

We initiated iron-oxidizing gradient cultures with the same groundwater samples that we used previously for direct molecular detection of iron-oxidizing *Gallionellaceae* (Hassan et al. 2015). We hypothesized a diverse range of cultivatable iron-cycling microorganisms to reside in arsenic contaminated groundwaters in Bangladesh. Indeed, all samples showed iron oxidation, and substantial diversity was found in these enrichments. As also hypothesized, we enriched microorganisms that had not been identified as iron oxidizers on the basis of our previous cultivation-independent 16S rRNA gene-based study (Hassan et al. 2015). However, as we did not isolate and characterize individual strains, it might be that not all identified microorganisms are capable of iron oxidation but live on the organic matter that is excreted by chemolithotrophic iron oxidizers (Ghiorse 1984). Nevertheless, since Fe(II) minerals were the sole source of electron donors in the enrichments and we targeted the dominant DGGE bands for phylogenetic analysis, we would expect to primarily encounter iron oxidizers.

Our culturing approach using opposing gradients of ferrous iron and oxygen is widely used to enrich and isolate *Gallionella* species (Emerson and Floyd 2005). Thereby, a striking finding was that the cultivation approach of the present study showed very limited persistence of *Gallionella*, whereas our prior study revealed that 77% of the 22 samples investigated contained *Gallionellaceae* (Hassan et al. 2015). The 16S rRNA gene of *Gallionella* could only be detected in 2 out of the 22 enrichment tubes, and only after using a *Gallionellaceae* specific PCR. Instead, a significant number of *Comamonadaceae*-related 16S rRNA gene sequences were detected. Similar findings were obtained by Blöthe and Roden (Blöthe and Roden 2009) in a cultivation-based study on groundwater seep material at circumneutral pH. Their 16S rRNA gene-based molecular analysis revealed that even though groundwater was dominated by *Gallionella* and *Leptothrix spp.*, they also could not isolate *Gallionella* and *Leptothrix spp.*, using the same opposing gradient cultivation method commonly used to enrich *Gallionella* (Emerson and Floyd 2005). Also several other studies have documented iron-oxidizing enrichments dominated by *Comamonadaceae* and *Rhodocyclaceae* rather than *Gallionella spp.* (Yu et al. 2010; Gülay et al. 2013). A possible explanation for the low encounter frequency of *Gallionella spp.* in our study might be a relatively lower abundance of *Gallionella* compared to other taxa in the groundwater samples, not allowing them to be recoverable in our dilution to extinction culturing approach. Another explanation might be that *Gallionella* was outcompeted by other microorganisms under the imposed culturing conditions, for example, because of a lower specific growth rate.

Based on our DGGE fingerprinting analysis, members of the *Comamonadaceae* were most frequently observed (11 out of 41 bands; 27% of total), especially *Hydrogenophaga* but also *Curvibacter* and *Acidovorax* species. Recently, Chan et al. (2014) isolated iron-oxidizing *Hydrogenophaga sp.* P101 and *Curvibacter sp.* CD03 retrieved from alluvial sediment, Rifle aquifer in Colorado (USA) by microaerophilic iron-oxidizing gradient tube enrichment. Besides *Hydrogenophaga*, we also detected a number of sequences most closely related to the genera *Leptothrix*, *Pseudomonas*, and members of the *Rhodocyclaceae* (*Dechloromonas*), which contain iron-

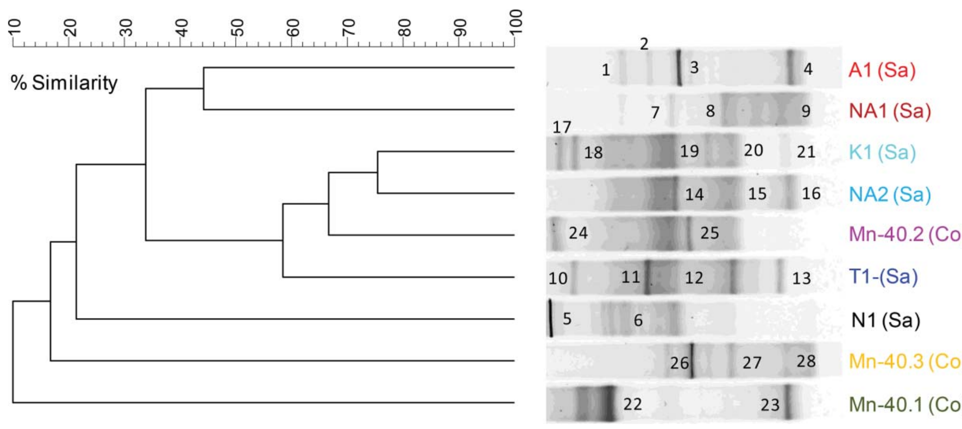

Figure 3. (a) UPGMA cluster analysis of bacterial 16S rRNA gene-based DGGE profiles (30–55% denaturant gradient) of nine iron reducing enrichments using Pearson correlation analysis to assess similarity. The enrichment ID refers to the location of the drinking water well (see Table 1). Numbers refer to the position of excised bands.

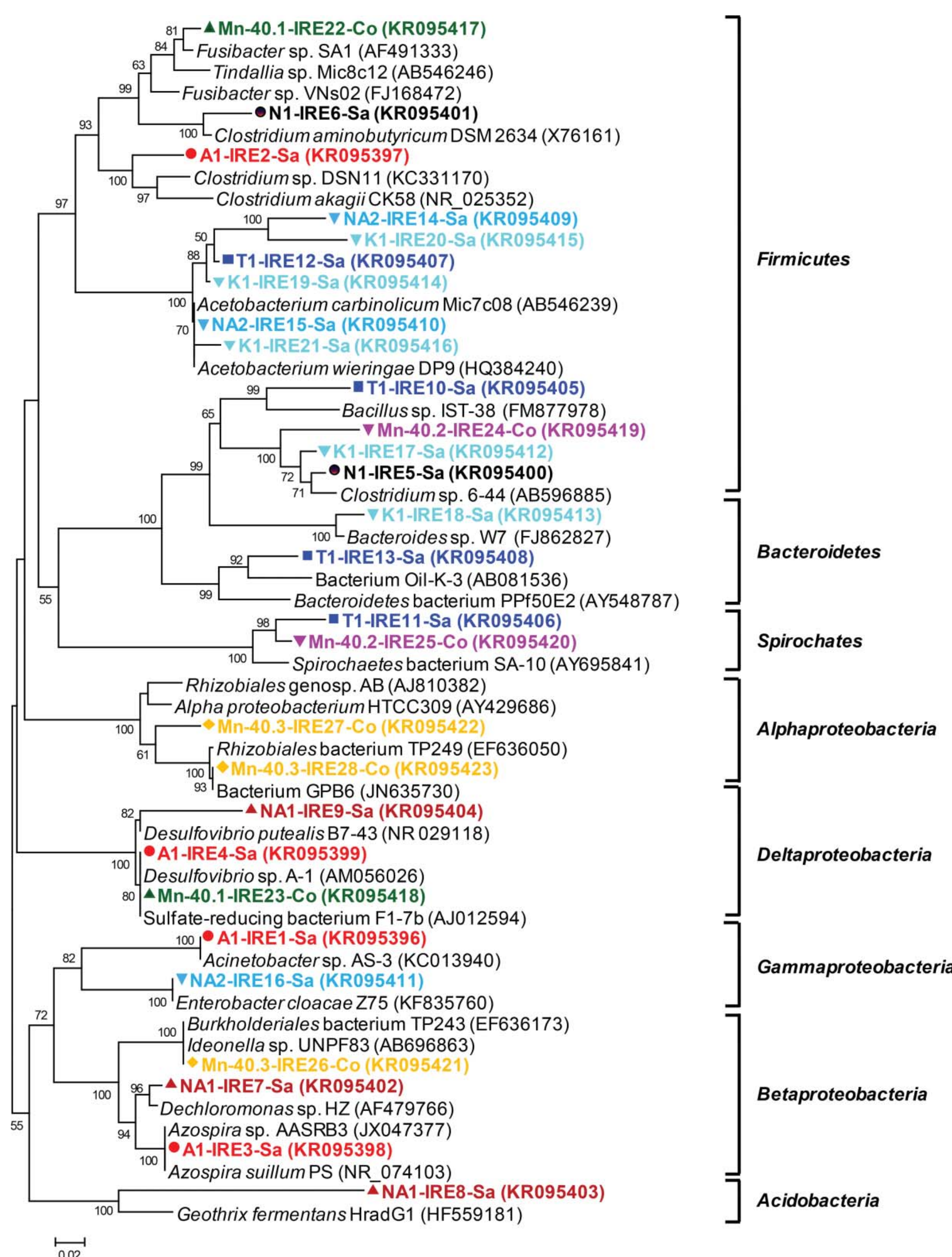

Figure 3. (b) Phylogenetic analysis of 16S rRNA gene sequences (525 unambiguously aligned nucleic acid positions) retrieved from the excised DGGE bands. Sequences are indicated by enrichment ID and the number of the excised band as shown in (a). Sequences are accompanied by a colored symbol, specific for each of the nine enrichments. The tree was constructed with the neighbor-joining method and bootstrap values (1000 replications) are indicated at the interior branches. The scale bar represents 2% sequence divergence.

oxidizing members (Hedrich et al. 2011), and to anaerobic denitrifying iron-oxidizing *Acidovorax* (Carlson et al. 2013). Similar sequences were also reported in our previous cultivation-independent study (Hassan et al. 2015). Several microorganisms are able to oxidize ferrous iron using nitrate or oxygen as electron acceptors (Yu et al. 2010). Many denitrifying bacteria are capable of switching to microaerophilic iron oxidation (Benz et al. 1998; Edwards et al. 2003; Melton et al. 2012), which may explain the occurrence of 16S rRNA genes most closely related to anaerobic denitrifying iron-oxidizing bacteria in the gradient tubes of the present study.

Forty-five percent of the iron-oxidizing enrichments harbored genes encoding *aioA*. This gene is either used by heterotrophs to detoxify arsenite or by chemolithoautotrophs to yield energy from arsenite oxidation (Santini et al. 2002; Inskeep et al. 2007; Rhine et al. 2007). We found only four AioA sequences in our iron-oxidizing gradient tubes that were most closely related to the corresponding sequences of chemolithoautotrophic strains, that is, *Ancylobacter dichloromethanicus* As3-1b (Andreoni et al. 2012), the nitrate reducing *Paracoccus sp.* SY (Zhang et al. 2015) and *Sinorhizobium sp.* DAO10 (Rhine et al. 2006). The other six sequences were most closely related to heterotrophs. Congruence was observed between *aioA* and 16S rRNA gene data, both indicating the presence of *Hydrogenophaga* and *Acidovorax* species. Some *Hydrogenophaga* species are known to be capable of aerobic arsenite oxidation (vanden Hoven and Santini 2004; Salmassi et al. 2006), while several *Acidovorax* strains perform nitrate-dependent iron (Klueglein

and Kappler 2013) and arsenite oxidation (Quéméneur et al. 2008). Also, some strains of *Dechloromonas sp.* oxidize iron (Weber et al. 2006b; Coby et al. 2011; Chakraborty and Picardal 2013) and arsenite under nitrate- or perchlorate-reducing conditions (Sun et al. 2009). However, to our knowledge prior studies did not investigate the presence of arsenite oxidase genes in iron-oxidizing enrichments, and overall our results indicate metabolic flexibility in several enrichments. The AioA sequences we identified were most closely related (>94% amino acid identity) to those identified on the basis of cultivation-independent analysis of the same water samples from which these enrichments were derived (Hassan et al. 2015).

Microbial diversity in iron-reducing enrichments

A substantial diversity was also found in the iron-reducing enrichments, including microorganisms that had not been identified as iron reducers on the basis of our previous cultivation-independent 16S rRNA gene-based study (Hassan et al. 2015). *Geobacteraceae* have often been demonstrated to constitute the most abundant iron reducers in iron-reducing subsurface environments (Lovley et al. 2011), including arsenic contaminated sediments in West Bengal, India (Islam et al. 2004; Héry et al. 2010). We encountered *Geobacteraceae* in all our enrichments. However, *Geobacter spp.* appeared to constitute a minor fraction of the communities observed in this study, in line with our previous cultivation-independent analysis of the same samples (Hassan et al. 2015). *Geobacteraceae* could only be revealed after specifically targeting their 16S rRNA genes. Enrichment recovered other and also more *Geobacter* phylotypes compared to our prior cultivation-independent analysis (Hassan et al. 2015).

Besides *Geobacter spp.*, other microorganisms can substantially contribute to iron reduction in iron-reducing environments (Lin et al. 2007; Li et al., 2011), and therewith affect the release of arsenic into groundwater (Rowland et al. 2009). Dominant phylotypes present in our iron-reducing enrichments belonged to the *Firmicutes*, a phylum also identified in our previous cultivation-independent study (Hassan et al. 2015). Several *Acetobacterium* sequences were observed, in addition to Clostridia and a sequence most closely related to *Bacillus sp.* strain IST-38. *Acetobacterium* species have been identified as anaerobic hydrogen consuming acetogens in iron corrosive settings (Mori et al. 2010), but iron reduction by *Acetobacterium* isolates has not yet been reported, to our knowledge. *Clostridium* 16S rRNA gene sequences were also retrieved during a cultivation-independent analysis of incubated arsenic contaminated West Bengal sediments, India (Islam et al. 2004). The capability of clostridia to reduce iron is well known. Subsurface iron-reducing *Clostridium* strains have for instance been isolated from a landfill leachate polluted aquifer in The Netherlands (Lin et al. 2007). They use ferric iron as an electron sink generating additional ATP via substrate level phosphorylation during acetate production (Lovley et al. 2011). Also *Bacillus sp.* strain IST-38, isolated from a groundwater seep in Alabama (USA), is capable of such fermentative iron reduction (Blöthe and Roden 2009).

Other sequences were also closely related to sequences of iron reducers, or sequences retrieved from iron-reducing environments. *Geothrix* is a dissimilatory iron-reducing bacterium, previously encountered in iron-reducing zones of hydrocarbon polluted aquifers and redox dynamic hydrocarbon polluted surface sediments (Coates et al. 1999; Klueglein et al. 2013). Sulfate reducing *Desulfovibrio* species were encountered in three iron-reducing culture enrichments (NA1, A1, and Mn-40.1). This is surprising perhaps; several *Desulfovibrio* species can enzymatically reduce iron, but do not conserve sufficient energy to support growth (Lovley et al. 1993). Active *Desulfovibrio* were recently identified in arsenic mobilizing Cambodian aquifer sediments (Héry et al. 2015). We identified a sequence most closely related to the fermentative iron-reducing *Bacteroides* strain W7 isolated from the anode suspension of a microbial electrolysis cell (Wang et al. 2010). Several sequences fell in the *Rhizobiales* order, a group not known to reduce iron. However, members of this group can reduce uranium (Vishnivetskaya et al. 2010). *Rhizobiales* were also abundant in anode biofilm communities that had been enriched for electricity generation (Ishii et al. 2008; Kaku et al. 2008). Interestingly, we encountered in the iron-reducing enrichments *Azospira* and *Dechloromonas*, which are known denitrifying iron-oxidizing *Betaproteobacteria*

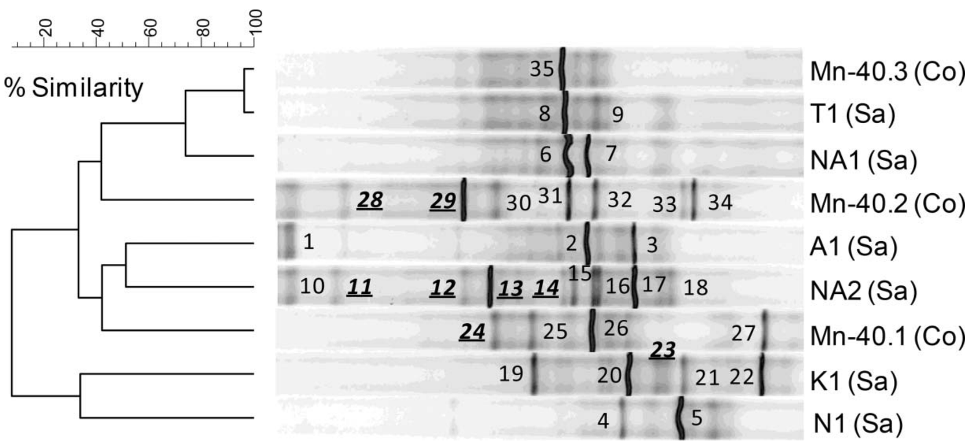

Figure 4. (a) UPGMA cluster analysis of *Desulfuromonadales* 16S rRNA gene-based DGGE profiles (30–55% denaturant gradient) of nine iron-reducing enrichments using Pearson correlation to assess similarity. The enrichment IDs refer to the location of the drinking water well (see Table 1 for details). Numbers refer to the position of excised bands subjected to sequencing. Sequences of bands with underlined, bold numbers in italics did not belong to *Desulfuromonadales*.

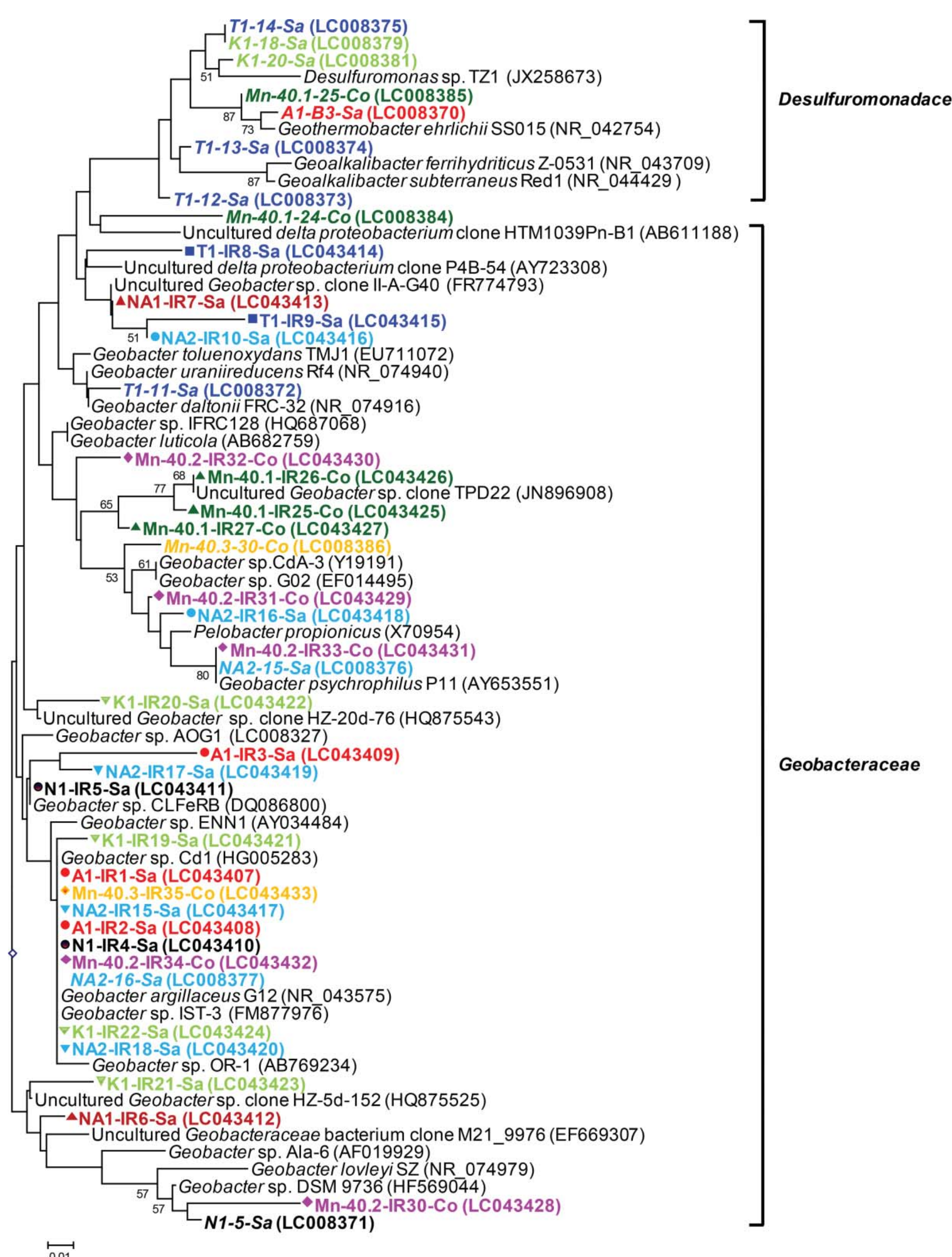

Figure 4. (b) Phylogenetic analysis of 16S rRNA gene sequences (122 unambiguously aligned nucleic acid positions) retrieved from the excised DGGE bands. Sequences are indicated by enrichment ID, the number of the excised band as shown in (a), and the district in which the well is located. Sequences are also accompanied by a colored symbol, specific for each of the nine enrichments and IDs in italics refer to water samples. The unrooted tree was constructed with the neighbor-joining method and bootstrap values (1000 replications) are indicated at the interior branches. The scale bar represents 1% sequence divergence.

(Weber et al. 2006a). *Dechloromonas* species have previously been isolated from an arsenic contaminated dimictic lake in Arlington, MA (Gibney and Nüsslein 2007) and nitrate reducing Wisconsin River sediment (Chakraborty and Picardal 2013), and *Azospira* from an animal waste lagoon (Byrne-Bailey and Coates 2012). These species were also encountered in anaerobic microbial iron-cycling reactors (Coby et al. 2011). We identified sequences of both *Dechloromonas sp.* and *Clostridium* in our iron-reducing enrichments that were >97% similar to those detected in the previous cultivation-independent 16S rRNA gene survey of potentially iron-cycling microbial communities (Hassan et al. 2015). Overall, the observations complement our previous findings (Hassan et al. 2015), confirming the abundance and diversity of microorganisms with potential for iron reduction in the investigated Bangladeshi aquifers. However, we cannot be completely certain that all microorganisms encountered in our enrichments are indeed capable of iron reduction, as we did not perform isolation and characterization of strains.

Some iron reducers can sometimes also reduce arsenate and possess the respiratory arsenate reductase gene *arrA* (Ohtsuka et al. 2013; Kudo et al. 2014; Osborne et al. 2015). *arrA* genes closely related to those of *Geobacter* species have been detected frequently in arsenic rich sediments (Lear et al. 2007; Héry et al. 2015). While some 44% of our iron-reducing enrichments held *arrA* genes, their sequences were more closely related to uncultivated species or *Sulfurospirillum* than to *Geobacter* species.

Table 2. Identities of amplified fragments of the arsenate respiratory reductase (*arrA*) gene (partial nucleotide sequences of 151 bp) detected in iron-reducing enrichments.

Enrichment ID	Accession numbers	Most closely related isolates	Nucleotide similarity (%)
N1	LC043403	Uncultured bacterium clone GW-1-56 (JN704765)	126/143 (88%)
		Geobacter uraniireducens Rf4 (CP000698)	72/99 (73%)
NA2	LC043404	*Sulfurospirillum barnesii* SES-3 (AY660884)	80/100 (80%)
		Uncultured bacterium clone GL-ARRA1 (EF014944)	115/150 (77%)
Mn-40.1	LC043405	Uncultured bacterium clone NP1_GW+G_20 (KF010035)	126/151 (83%)
Mn-40.2	LC043406	Uncultured bacterium clone GL-ARRA1 (EF014944)	148/150 (99%)

Implications for the (im)mobilization of arsenic

The mobilization of arsenic in subsurface environments has enormous toxic consequences for millions of people in Bangladesh who are vulnerable through their use of groundwater as drinking water and through arsenic entering the food chain (Huq et al. 2006). Currently available remediation technologies have major disadvantages such as they are often expensive and result in secondary exposure to and environmental pollution with arsenic, for example, through inadequate handling and disposal of arsenic binding water filters (Gonzaga et al. 2006). The nature of the minerals formed during microbial iron oxidation may have important implications for arsenic mobility. Biological ferrous oxidation can effectively sequester arsenic via the precipitation of ferric oxyhydroxide minerals (Hohmann et al. 2009). Several microbial iron oxidizers produce poorly soluble crystalline iron oxides (Miot et al. 2009; Liu et al. 2013; Li et al. 2015). Biogenic amorphous iron oxides play a major role in removing arsenite due to their strong capacity to adsorb or coprecipitate arsenite (Omoregie et al. 2013). With respect to microaerophilic iron oxidation, most attention has been focused on *Gallionella* (Hedrich et al. 2011). Its role and effectiveness in arsenic removal from water by producing arsenic adsorbing ferric (oxyhydr)oxides has been established (Katsoyiannis and Zouboulis 2004; Zouboulis and Katsoyiannis 2005). Recently, it was shown that *Citrobacter freundii* strain PXL1 could remove arsenite from water in a sewage plant, in a process associated with iron oxidation (Li et al. 2015). The *Comamonadaceae* and *Rhodocyclaceae* identified here in many gradient tube enrichments may also be of interest with regard to biological iron and arsenic removal, in particular since consortia containing these bacteria revealed the presence of *aioA* genes. Arsenite oxidases oxidize arsenite to the less toxic and less mobile arsenate. This potential for ferric (oxyhydr)oxides precipitation and arsenic immobilization makes these iron-oxidizing enrichments of interest for bioaugmentation of reactors treating arsenite contaminated groundwater retrieved from drinking water wells. They may also be of interest for *in situ* removal of arsenic and iron by subsurface arsenic removal (SAR) technology (van Halem et al. 2010). SAR was recently introduced in Bangladesh. It comprises the injection of oxygenated water into aquifers so as to oxidize ferrous iron abiotically and therewith precipitate iron and adsorb and coprecipitate arsenic (van Halem et al. 2010). The activity of iron-oxidizing microorganisms that also oxidize arsenite could potentially enhance SAR efficiency down to lower residual concentrations of arsenic.

Whether biological arsenic removal from groundwater in Bangladesh would be conducted *in situ* or *ex situ*, care should be taken to avoid anaerobic conditions. Groundwater in Bangladesh also contains a wide range of anaerobic iron reducers (Hassan et al. 2015, this study), which may then become active and release arsenic as a result of the reduction of precipitated and arsenic adsorbing ferric (oxyhydr)oxides. Specialized arsenate reducers and iron reducers possessing *arrA* (Hassan et al. 2015, this study) may enhance the release of arsenic by reducing arsenate to more mobile and more toxic arsenite.

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