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Phylogenetic analysis and arsenate reduction effect of the arsenic-reducing bacteria enriched from contaminated soils at an abandoned smelter site

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Abstract

Microbial reduction of As(V) (i.e., arsenate) plays an important role in arsenic (As) mobilization in aqueous environment. In this study, we investigated As(V) reduction characteristics of the bacteria enriched from the arsenic-contaminated soil at an abandoned smelter site. It was found that As(V) was completely reduced to As(III) (i.e., arsenite) in 21 h. After 3-d incubation, a yellow solid was precipitated and the concentration of As(III) decreased sharply. After 150 h incubation, ca. 65% of soluble arsenic was removed from the solution. The analysis of the precipitate by scanning electron microscopy and energy dispersive spectrometer (SEM-EDS) and X-ray diffraction (XRD) revealed that the main component was crystalline arsenic sulfide (AsS). Microbial mediated reduction and mobilization of adsorbed As(V) on ferric hydroxide was also examined. In the microcosm slurry experiment, ca. 53% of the adsorbed As(V) was reduced to As(III) by the bacteria, which resulted in an appreciable release of arsenic into aqueous phase. The released arsenic was present predominantly as As(III). The microbial diversity was analyzed by 16S rDNA-dependent molecular phylogeny. A near-full-length 16S rDNA gene clone library was constructed. The 197 clones were analyzed using RFLP (restriction fragment length polymorphism) and 72 OTUs were obtained, which contributed 51% of the content for total clone number in six OTUs. Six bacterial clones in these six OTUs were selected for sequencing and the sequenced clones were found to belong to the group *Caloramator*, *Clostridium*, and *Bacillus*.

Key words: microbial; reduction; arsenic; phylogenetic analysis

Introduction

Arsenic (As) is toxic to humans, animals and plants. It is a strong carcinogen to humans and can cause skin and internal organ cancers as well as other diseases such as skin lesions, hyperkeratosis, melanosis. Arsenic contamination occurs worldwide and the areas with high-arsenic groundwater have been reported in Argentina, Chile, China, India, Bangladesh, and Vietnam and so on. In Bangladesh, one-third of the estimated 9–11 million tubewells deliver groundwater with geogenic arsenic concentrations above 50 μg/L and put 30–40 million people at risk of chronic arsenic poisoning (Smedley and Kinniburgh, 2002). WHO guideline value for arsenic in drinking water was reduced from 50 to 10 μg/L in 1993 (WHO, 1993). The US Environmental Protection Agency had also set the drinking water standard for arsenic at 10 μg/L, which was put into effect for the public water systems to comply with by January, 2006 (USEPA, 2001).

As(V) and As(III) comprise the bulk of the inorganic speciation encountered in aqueous environment (Cullen and Reimer, 1989). Arsenate is the major arsenic form in oxidizing environments, whereas in reducing environments arsenic occurs predominantly as arsenite. It was reported that arsenite is 25–60 times more toxic and more mobile than arsenate under most environmental conditions (Smedley and Kinniburgh, 2002). Therefore, in arsenic-contaminated environments, a major concern is the potential for the reduction of As(V) to As(III), which may initiate the mobilization of arsenic in aqueous environments.

Microbes play an important role in arsenic's geochemical cycle. Under anaerobic conditions, biogenetic As(III) could be achieved from two microbiological reductive mechanism. As(V) can be directly reduced to As(III) as the terminal electron acceptor by the microbes, yielding energy to support their growth and defined as dissimilatory arsenate reducing prokaryotes (DARPs). On the other hand, microbes could reduce As(V) via arsenic detoxification and resistant mechanism but could not obtain energy. Dissimilatory arsenate reduction may enhance the solubility of arsenic, particularly in environments with low iron (hydr)oxide content. Zobrist *et al*. (2000), for example, reported that microbial reduction of As(V) adsorbed or coprecipitated on ferric and aluminum hydroxides greatly increased dissolved As(III) concentration. Arsenate-respiring bacteria and archaea have recently been

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isolated from a diversity of natural environments, including freshwater streams and sediments, alkaline and saline lakes, and hot springs, and had been investigated in detail (Stolz and Oremland, 1999; Huber *et al*., 2000; Mukhopadhyay *et al*., 2002; Oremland and Stolz, 2003).

The objectives of this study were: (1) to investigate the reduction characteristics of both aqueous and adsorbed arsenate on ferric hydroxide by culturable anaerobic bacteria enriched from the arsenic-contaminated soil at an abandoned smelter site; (2) to determine arsenic speciation in both aqueous and solid phases after reaction; (3) to identify the microbial populations by using the 16S rDNA sequence analysis.

1 Materials and methods

1.1 Abandoned smelter site

The abandoned smelter site is located in Shenyang, northeast of China, with an area of 0.36 km^2. It was operated from 1936 to 2001 for the production Cu, Pb, Zn, Au, Ag and so on. The 65 years of metallurgical operation resulted in the accumulation of high level of arsenic in the surrounding soils, most of which was over 600 mg/kg. The soil sample used in the experiment was collected at 10–30 cm depth beneath the surface in March, 2006.

1.2 Bacterial enrichment cultivation

The soil sample 1.0 g was placed into a sterile, 20-ml anaerobic tube with 10 ml of distilled water. The anaerobic tube was vigorously mixed with a vortex mixer, 1 ml of the resulting suspension was inoculated into the anoxic minimal salts medium containing 10 mmol/L lactate, 75 mg/L sodium arsenate ($Na_3AsO_4{\cdot}12H_2O$), with 0.05% yeast extract as nutrient supplement and *L*-cysteine (0.4 g/L) as the reducing agent. The salts used (g/L) were: KH_2PO_4 (0.14), NH_4Cl (0.25), KCl (0.5), $CaCl_2$ (0.113), NaCl (1.0), and $MgCl_2{\cdot}6H_2O$ (0.62). The added trace minerals were (mg/L): $MnCl_2{\cdot}4H_2O$ (0.1), $CoCl_2{\cdot}6H_2O$ (0.12), $ZnCl_2$ (0.07), H_3BO_3 (0.06), $NiCl_2{\cdot}6H_2O$ (0.025), $CuCl_2{\cdot}2H_2O$ (0.015), $Na_2MoO_4{\cdot}2H_2O$ (0.025), and $FeCl_2{\cdot}4H_2O$ (1.5). The final pH of the media was adjusted to 7.0 by adding 0.1 mol/L NaOH solution. The mixture was incubated in the dark at 30°C. After a week enrichment, 0.4 ml of the initial slurry was transferred to another fresh 10 ml anoxic minimal salts medium, and incubated for another week. After several times of such transfers and incubations, a stable enrichment culture was obtained.

All incubations were performed in 20 ml pressure tubes sealed with butyl rubber stoppers and screw-cap at 30°C in the dark unless otherwise specified. And *L*-cysteine was added before inoculation. All preparations and manipulations were performed under strict anoxic conditions using the Hungate technique (Hungate *et al*., 1969). All transfers were made using needles and syringes.

1.3 Microbial reduction of aqueous As(V)

The ability of microbial reduction of aqueous As(V) was tested by inoculating the enriched bacteria into the anoxic minimal salts medium as described above. The initial concentration of As(V) was kept at 75 mg/L also. The controlled experiment was carried out at the same conditions without inoculation. All incubations, preparations and manipulations were performed under the same conditions described above.

1.4 Microbial reduction of As(V) adsorbed on ferric hydroxide

Ferric hydroxide was synthesized in the laboratory by fast hydrolysis of $FeCl_3$ solution (0.1 mol/L) with 0.5 mol/L NaOH as described by Schwertmann and Cornell (1991). The slurry was stabilized at pH 8 for 1 h after neutralization. The pH of the slurry was adjusted to 7.3 and maintained at that pH for 1 h prior to adsorption experiments. Arsenate solution of pH 7.3 was introduced into the ferric hydroxide slurry at the molar ratio of Fe/As = 8 and the pH of the mixture was controlled constant at pH 7.3 by addition of NaOH and/or HCl solution. The adsorption system was equilibrated for 24 h under constant stirring. The arsenate adsorbed ferric hydroxide was separated by centrifuging and resuspended in 500 ml distilled water.

Five milliliter of the slurry and 20 ml of 1.25× concentrated anoxic minimal salts media were added to the 50-ml bottle with a butyl rubber stopper and a screw-cap lid to achieve an As concentration of 160.5 mg/L in suspension. Reducing agent such as *L*-cysteine was not used in this case. The suspensions were allowed to spurge with ultrapure N_2 to minimize O_2 uptake. The capped serum bottles were autoclaved, inoculated and incubated on a rotary shaker (170 r/min) at 33°C in the dark. In each experiment, two additional bottles were left uninoculated as a control and sampled in an identical manner.

1.5 Analytical techniques

The potential of microbial arsenic reduction was investigated in the enriched bacterial culture where As(V) existed as the free oxyanion. Five milliliter of suspension was removed from the anaerobic tube to filter through a 0.22-μm pore-size membrane filter. As(III) and As(T) (total As) were analyzed in the filtrate.

For the slurry incubations, at select time points, the serum bottles were removed from the incubator and transferred to an anaerobic (ultrapure N_2) glove chamber. 5 ml of suspension was sampled and filtered through a 0.22-μm pore-size membrane filter. This fraction was considered to be the dissolved fraction and analyzed for As(III) and As(T). Total concentration of As(III) was obtained by placing 2.5 ml of suspension directly into 2.5 ml of 1 mol/L HCl, mixing, and allowed to stand for at least 1 h before analysis.

The concentration of As(III) and As(T) were determined on a Varian AA-240 atomic absorption spectrometer coupled with a hydride generator. To detect As(III) selectively, the working solution was prepared in a pH 4.4 sodium citrate buffer (0.4 mol/L), with a mixture of 0.8% KBH_4 and 0.2% NaOH as reducing solution and 0.1 mol/L citric acid as carrier solution. Under this condition, only As(III)

was converted to AsH_3 and detected with atomic absorption spectrum instrument (Maity *et al*., 2004; Quináia and Rollemberg, 2001). The concentration of As(T) was determined by treating the solution with a reducing agent containing 5% thiourea and 5% ascorbic acid prior to hydride generation and AAS measurement, using a solution containing 1.5% KBH_4 and 0.3% NaOH as reducing solution, and 1% HCl as carrier solution.

The precipitate was characterized by an Oxford EDX analyzer and a Rigaku D/max 2000PC X-ray diffractometer (M/S. Rigaku Corporation, Japan) operating at a voltage of 50 kV and a current of 300 mA with Cu K_α radiations.

1.6 DNA extraction

Total DNA was extracted from the enriched culture according to the protocol described in "Short protocols in molecular biology" (Ausubel *et al*., 2005). Briefly, 5 ml of the culture sample was centrifuged for 2 min. The bacterial pallets were resuspended with 570 μl of Tris-EDTA buffer (0.01 mol/L Tris, 0.001 mol/L EDTA, pH 8.0) and incubated at 37°C for 1 h after adding 30 μl of 10% sodium dodecyl sulfate (SDS) and 3 μl of proteinase K. Moreover, the DNA solution was further incubated at 65°C for 10 min after addition 100 μl of 5 mol/L NaCl and 80 μl of CTAB/NaCl, followed by separation with phenol-chloroform-isoamylalcohol (25:24:1). After centrifuging at 12,000 r/min, the supernatant was transferred to a tube and separated with chloroform-isoamylalcohol (24:1) again. The supernatant was transferred to another tube and the total DNA was precipitated by adding 0.6 volume of isopropanol and leaving to stand still for 2 h at 4°C, followed by centrifuging for 20 min at 14,000 r/min at 4°C. The DNA pellet was washed with 70% ethanol, centrifuged for 20 min at 14,000 r/min at 4°C, dried at room temperature, then resuspended in 50 μl of TE buffer. The DNA purity and size distribution (predominantly ca. 23 kb) were estimated by electrophoresis in a 1.0% agarose Tris-acetate-EDTA (TAE) gel and viewed by GoldViewTM staining.

1.7 PCR and cloning of 16S rDNA gene

The extracted DNA was used as the template for PCR amplification. The near-full-length 16S rRNA gene was amplified using eubacterial primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGTTACCTTGTTACGACTT-3'). The PCR protocol is: initial denaturation at 95°C for 5 min, melting at 94°C for 1 min, annealing at 48°C for 1 min, extending at 72°C for 2 min, 31 cycles, with a final extension step at 72°C for 10 min. The amplified product was analyzed by electrophoresis in a 1.0% agarose Tris-acetate-EDTA (TAE) gel. The band of the proper size range (ca. 1,500 bases) was excised and purified with a TaKaRa Agarose Gel DNA Purification Kit Ver.2.0 (TaKaRa, Japan). The purified products were ligated directly into the cloning pMD19-T simple vector (TaKaRa, Japan) according to the introduction provided by the manufacturer before transformation into *Escherichia coli* DH5α competent cells. White transformants that grew on LB agar containing ampicillin (100 μg/ml), IPTG (24 μg/ml) and X-Gal (40 μg/ml) were screened. Plasmid DNA was prepared by heat lysis. The cloned 16S rDNA gene inserts were effectively amplified with a vector-specific primer set of the pMD19-T simple vector (TaKaRa, Japan) RV-M (5'-GAGCGGATAACAATTTCACACAGG-3') and M13-47 (5'-CGCCAGGGTTTTCCCAGTCACGAC-3'). The PCR method was: initial denaturation at 95°C for 5 min, melting at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min; 31 cycles, with a final extension step at 72°C for 10 min. The PCR products were analyzed by agarose electrophoresis gel as described above and those clones containing correct DNA insertion were kept.

1.8 Restriction fragment length polymorphism (RFLP) and phylogenetic analysis

Each PCR amplified product in the chosen clones was firstly treated with restriction endonucleases *Taq* I. The restriction enzyme digests were separated using a 10% polyacrylamide TAE gel and viewed by GeneFinderTM staining. The digests that had the same restriction patterns were further analyzed with *Hinf* I restriction endonucleases and were determined by polyacrylamide gel as described above. After electrophoresis, the bacterial clones were categorized manually into different operational taxonomic unit (OTU) based on the restriction patterns. A RFLP clone in each of OTUs was selected for sequencing, and the sequences were compared with the published sequences in GenBank using BLAST program packages. Phylogenetic tree was constructed using the neighbor-joining method.

2 Results and discussion

2.1 Microbial reduction of aqueous arsenate

The concentration of soluble As(T) and As(III) over cultivation time was monitored (Fig.1). The result indicates that cell suspensions quantitatively reduced As(V) to As(III). All the added As(V) (ca. 75 mg/L) was completely reduced to As(III) in 21 h. After 21 h, the curve of As(III) overlapped with that of As(T), indicating that no As(V) was present and all the soluble arsenic existed as As(III).

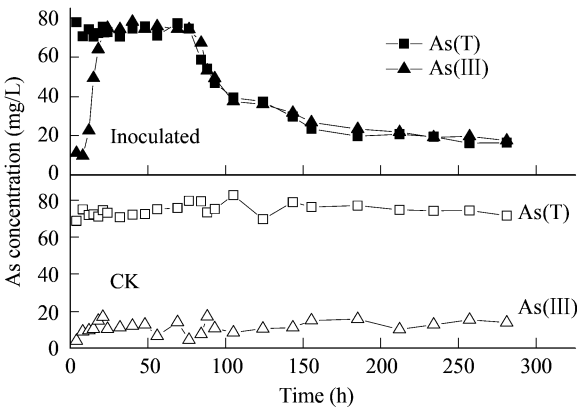

Fig. 1 Change of the concentrations of dissolved As(III) and As(T) over the incubation time. Initial As(V) concentration was 75 mg/L. Top: inoculated; bottom: uninoculated (CK).

The concentration of arsenic remained nearly unchanged in next ca. 2 d. After ca. 3 d incubation, the concentration of arsenic declined rapidly, which was accompanied by the precipitation of a yellow solid. Approximately 65% of soluble arsenic was removed from the medium as arsenic sulfide (AsS) after 6 d incubation. The concentration of arsenic changed little thereafter during prolonged incubation.

In the controls without inoculation, arsenate was slightly reduced (Fig.1). As(III) concentration was ca. 11.5 mg/L while As(T) concentration was constant at ca. 75 mg/L, suggesting that no arsenic was removed from the medium and AsS precipitation did not occur in the abiotic controls. The slight reduction of arsenate was probably due to the addition of *L*-cysteine which can reduce the arsenate under low pH.

Yellow precipitate was observed after 3 d incubation. After more than 10 d cultivation, the precipitate was collected and characterized by EDS (energy dispersive spectrometer) in conjunction with SEM (scanning electron microscope). The EDS analysis showed that the precipitate composed of O, As and S and revealed that the chemical composition of the precipitated sample was 32.4% O, 32.2% S and 35.4% As by atomic number (Fig.2). The molar ratio of As/S was found to be ca. 1.

X-ray diffraction (XRD) analysis of the precipitate (Fig.3) clearly revealed that the precipitate consisted predominantly of crystalline AsS which exists as at least three different crystalline phases. The XRD patterns of the sample corresponded to AsS (PDF cards no. 24-0078 and 25-0057) and reaglar (PDF cards no. 09-0441). No crystalline compounds containing C or/and O were detected from the XRD patterns. The presence of the C and O peaks on the EDS profiles is due to the bacteria and organic compound such as glucide. In this experiment, As and S came from arsenate and *L*-cysteine, respectively. Apparently the arsenic sulfide was formed as a result of bioreduction of As(V). Some researchers suggested that sulphate-reducing bacteria promote the deposition of arsenic as sulfide minerals via their production of sulfide (Sracek *et al*., 2004). Kirk *et al*. (2004) also provided evidence that sulfate-reducing bacteria hold arsenic levels low for the sulfide produced possibly reacts to precipitate arsenic, or coprecipitate it with iron. Newman *et al*. (1997) reported that a sulfate-reducing bacterium, *Desulfotomaculum auripigmentum*, that can also reduce arsenate, had the ability to precipitate As_2S_3 both intra- and extracellularly. Furthermore, our result indicated that arsenic-reducing bacteria could decrease the mobility of arsenic by forming the precipitate of arsenic sulfide.

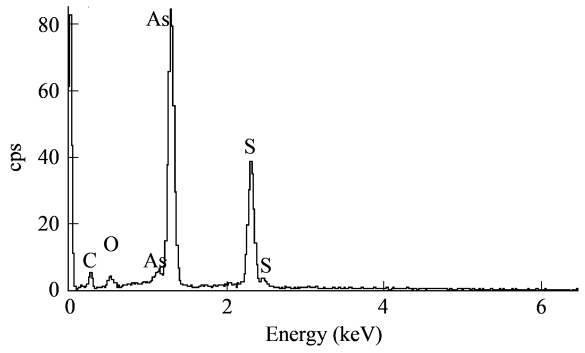

Fig. 2 Energy dispersive microanalysis of the precipitated solid during cultivation of the bacteria in 75 mg/L As(V) solution.

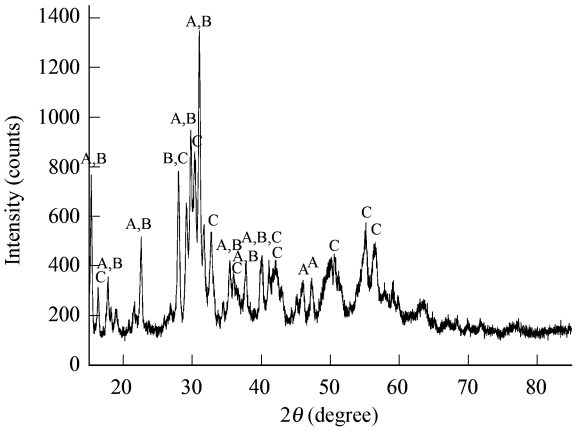

Fig. 3 X-ray diffraction patterns of the precipitated solid during cultivation of the bacteria in 75 mg/L As(V) solution. (A) arsenic sulfide (PDF# 24-0078); (B) alacranite (PDF# 25-0057); (C) realgar (PDF# 09-0441).

2.2 Microbial reduction of adsorbed As(V) on ferric hydroxide

Adsorption of arsenate on ferric hydroxide is an important process controlling the mobility and fate of arsenic in environment. The reduction and release of adsorbed As(V) on ferric hydroxide by the enriched bacteria was investigated and the results are shown in Fig.4. The concentration of arsenic in the solution of uninoculated system was ca. 3 mg/L and remained constant over the incubation process. The arsenic was present as As(V) since no As(III) was detected either in solution or in the solid, which indicates that reduction reaction did not occur. In comparison, arsenate reduction occurred in the inoculated system and the adsorbed arsenic was slightly released into the solution (Fig.4). The concentration of As(III) in the slurry increased sharply in the first several days of incubation and reached ca. 85 mg/L after 20 d, which represented

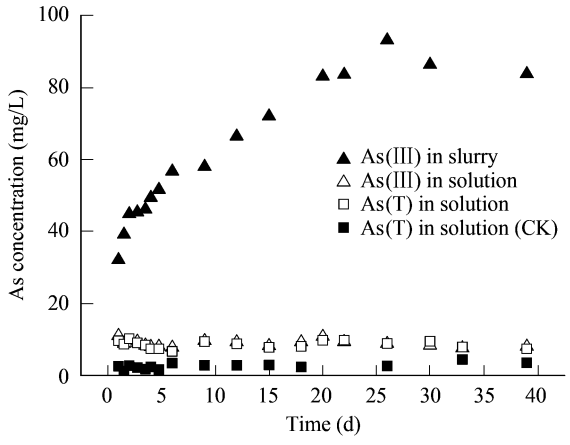

Fig. 4 Microbial reduction and release of adsorbed arsenate on ferric hydroxide. Initial As(V) concentration in slurry was 160.5 mg/L and the Fe/As molar ratio was ca. 8.

ca. 53% of original As(V) concentration (i.e., 160.5 mg/L). Aqueous arsenic was almost completely present in the form of As(III) and its concentration remained by and large constant at ca. 9 mg/L over the incubation process. This was 3 times higher than that of the uninoculated system, suggesting that the adsorbed arsenic was slight remobilized as the result of microbial reduction. This result also indicated that about 90% of As(III) reduced from arsenate under this condition was readsorbed to the surface of ferrihydrite.

It was reported that microbial reduction of iron could promote the formation of magnetite (Fredrickson *et al*., 1998; Zachara *et al*., 2002; Hansel *et al*., 2003; Islam *et al*., 2005). Iron reduction was observed in the inoculated groups, however, XRD characterization of the resultant solid after incubation could not reveal the presence of magnetite owing to the amorphous nature of the material. Coker *et al*. (2006) used X-ray absorption spectroscopy (XAS) and X-ray magnetic circular dichroism (XMCD) to elucidate the change of the bonding mechanism of As(III) and As(V) as bacteria induced reductive transformation to magnetite. The result showed that As(V) was incorporated within the magnetite structure; in comparison, As(III) was simply adsorbed to the surface of ferrihydrite. This is likely the reason why As(III) was released after microbial reduction although As(III) and As(V) were adsorbed to a similar extent on ferric hydroxide at neutral pH (Raven *et al*., 1998; Dixit and Hering, 2003). Furthermore, the transformation of ferric hydroxide to magnetite decreases the surface area of the solid, which may also result in the release of As(III).

2.3 Phylogenetic analysis of the enriched bacterial community

In the experiment, we constructed a 16S rDNA clone library for describing the phylogenetic diversity of subsurface microorganisms in anaerobic environments. Approximately 1.5-kb portions of bacterial 16S rDNA gene fragments from the enriched culture were amplified. A clone library was constructed, and 197 clones were analyzed. The 16S rDNA gene clones were divided into 72 OTUs based on RFLP. The clone numbers in six OTUs covered 51% of the total numbers. 8.6% of clones were contained in RFLP group z1, 9.1% in group z2, 9.6% in group z3, 5.6% in group z4, 11.2% in group z5, and 6.6% in group z6. For each of these six OTUs, the 16S rDNA insert of one representative clone was selected to be sequenced. The near-full-length 16S rDNA sequences were obtained after deleting the vector DNA sequences and were used to search the GenBank nucleotide database with the BLAST search tool. None of the bacterial sequences had a 100% similarity to sequences published in various data banks. However, the sequences were closely associated with published sequences and had a similarity above 92%. The phylogenetic analysis (Fig.5) suggested that these OTUs belonged to the classes *Caloramator*, *Clostridium*, and *Bacillus*.

The concentration of arsenic in the environment is much lower than that used in our experiment. The high concentration of arsenic probably decreased microbial population and phylogenetic diversity. The number of culturable bacteria should be much less than that of the

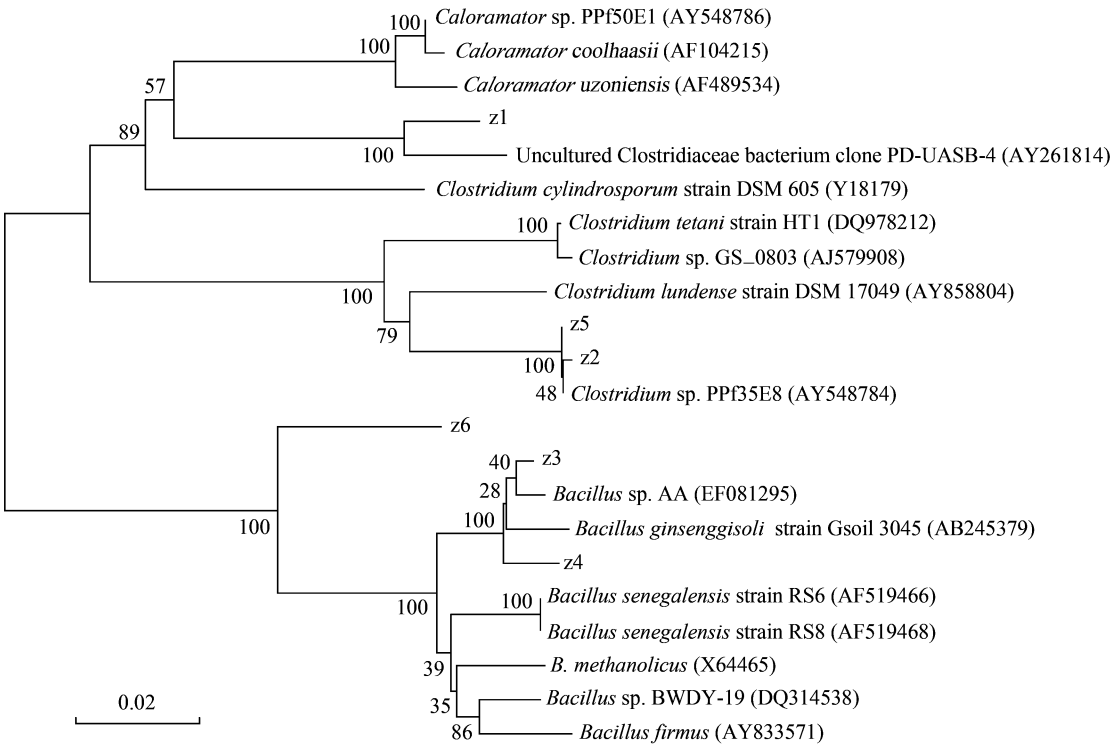

Fig. 5 Phylogenetic distance tree based on the comparative analysis of partial 16S rRNA sequences (approximately 1.5 kb) recovered from the enriched culture.

unculturable bacteria. Hence, only a fraction of the arsenic-resistant bacteria was included in this clone library. The actual genetic diversity of the anaerobic arsenic resistant microorganisms was probably much higher than 72 OTUs. Hence it may be inferred that arsenic-resistant mechanisms may be very widespread in natural environment.

Microbial reduction of arsenic plays a very important role in biogeochemical cycling of arsenic. It was reported that some arsenate respiring bacteria have been isolated from different environments, including sediments, alkaline and saline lake, and hot springs. These anaerobic bacteria were thought to be responsible for arsenic release to the environment and the increase of arsenic mobility (Ahmann *et al*., 1994, 1997; Oremland and Stolz, 2003; Islam *et al*., 2004). In the present work, we have enriched the bacteria from the arsenic contaminated soil at an abandoned smelter site, which has the ability to reduce As(V) to As(III) under anaerobic conditions at arsenic concentration as high as 75 mg/L. The findings may have the following environmental relevance: first, because arsenic is usually disposed in metallurgical tailings as arsenate, the bacterial activity at the abandoned smelter site may initiate the reduction of As(V) to As(III), as a result, causing mobilization and transport of the arsenic in highly contaminated soil and tailings to surrounding surface water and groundwater; second, the bacteria obtained in this study can reduce As(V) to As(III) at arsenic concentration as high as 75 mg/L, hence may be used in microbial treatment of arsenic-bearing industrial effluents. It was reported that arsenic can be removed from wastewater by microbial reduction and precipitation of arsenic sulfide. Hydrometallurgical solutions and wastewaters usually contain very high concentration of arsenic (in the form of arsenate), from several tens mg/L to several thousands mg/L. The bacteria used in this work provided a potential candidate for industrial arsenic-removal operations.

3 Conclusions

Arsenate reducing bacteria were isolated from the arsenic-contaminated soil at an abandoned smelter site and the microbial diversity was analyzed by 16S rDNA-dependent molecular phylogeny. The reduction of both aqueous and adsorbed arsenate on ferric hydroxide by the bacteria was investigated. The results showed that aqueous arsenate (concentration up to 75 mg/L) could be completely reduced to arsenite very quickly. Much of the soluble As(III) could be precipitated as arsenic sulfide in the presence of sulfide. More than half of the adsorbed arsenate on ferric hydroxide (Fe/As = 8) was reduced to arsenite, which caused a slight release of arsenic into aqueous phase.

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