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ScienceDirect
Journal of Environmental Sciences 2011, 23(9) 1544–1550
JOURNAL OF ENVIRONMENTAL SCIENCES
ISSN 1001-0742
CN 11-2629/X
www.jesc.ac.cn

Arsenic removal from contaminated soil via biovolatilization by genetically engineered bacteria under laboratory conditions

Shuang Liu[1], Fan Zhang[2], Jian Chen[1], Guoxin Sun[1,*]

1. State Key Laboratory of Urban and Regional Ecology, Research Center for Eco-Environmental Sciences, the Chinese Academy of Sciences, Beijing 100085, China. E-mail: liushuanghappy365@yahoo.com.cn
2. Institute of Plant and Environmental Protection, Beijing Academy of Agriculture and Forestry Science, Beijing 100089, China

Received 01 November2010; revised 10 December 2010; accepted 18 January 2011

Abstract

In *Rhodopseudomonas palustris*, an *arsM* gene, encoding bacterial and archaeal homologues of the mammalian Cyt19 As(III) S-adenosylmethionine methytransferase, was regulated by arsenicals. An expression of *arsM* was introduced into strains for the methylation of arsenic. When *arsM* was expressed in *Sphingomonas desiccabilis* and *Bacillus idriensis*, it had 10 folds increase of methyled arsenic gas compared to wild type in aqueous system. In soil system, about 2.2%–4.5% of arsenic was removed by biovolatilization during 30 days. This study demonstrated that arsenic could be removed through volatilization from the contaminated soil by bacteria which have *arsM* gene expressed. These results showed that it is possible to use microorganisms expressing *arsM* as an inexpensive, efficient strategy for arsenic bioremediation from contaminated water and soil.

Key words: genetic engineering bacteria; volatile arsenic; biovolatilization; bioremediation

DOI: 10.1016/S1001-0742(10)60570-0

Citation: Liu S, Zhang F, Chen J, Sun G X, 2011. Arsenic removal from contaminated soil via biovolatilization by genetically engineered bacteria under laboratory conditions. Journal of Environmental Sciences, 23(9): 1544–1550

Introduction

Arsenic (As) is an extremely toxic metalloid widely distributed in soils and water. Additional anthropogenic source of mining activities, utilization of arsenic-based pesticides or herbicides, and irrigation with arsenic contaminated water caused heavy contamination of soil, especially in farmland ecosystem (Williams et al., 2009; Jackson et al., 2006; Peryea and Kammereck, 1997; Chen et al., 2008). Arsenic accumulated in edible parts of plants could get into body through food chain and pose a serious health risk to humans (Yan, 1994; Abedin et al., 2002). Arsenic is known to have mutagenic and genotoxic effects on humans, and it has been associated with increased risk of skin, kidney, lung, and bladder cancers (Karagas et al., 1998). Thus, there is an urgent need to efficiently remove arsenic from contaminated water and soil.

Bioremediation of arsenic by microorganisms has been widely hailed because of their potential advantage in providing a cost-effective technology and environmental friendly way for heavy-metal removal (Valls and Lorenzo, 2002). Biovolatilization of arsenic is a natural process in the environment by which arsenic is lost from soil or water (Jakob et al., 2010), and can also be considered to be a bioremediation tool (Cox, 1973). The formation of volatile arsenic is an enzymatic process of organic and inorganic arsenicals through a reduction of As(V) to As(III) and a series of methylation reactions (Cullen and Reimer, 1989; Michalke et al., 2000; Mukhopadhyay et al., 2002). Arsenicals in the soil could be converted into their volatile derivatives by soil microbes and lost. There are many reports about arsenic volatilization by microfloras in soil or bacterial species in pure culture. Both aerobic and anaerobic microorganisms such as bacteria and microscopic fungi are particularly responsible for the evolution of volatile arsenicals (Cullen and Reimer, 1989; Cernansky et al., 2009; Thomas and Rhue, 1997; Friedrich and Merkel, 2006; Prohaska et al., 1999; Turpeinen et al., 2002; Edvantoro et al., 2004; Meyer et al., 2008). However, previous publications have shown that the rate of arsenic volatilization by soil microbial communities were limited (Rodriguez, 1998).

The amount of arsenic volatilized by microorganisms was governed by many factors such as arsenic forms and concentration, soil moisture, temperature, organic materials, other elements, microbial growth and capacity of arsenic volatilization (Cox, 1973; Edvantoro et al., 2004; Gao and Burau, 1997). The improvement of arsenic methylating ability of microbes by genetic engineering method could be a potential strategy to enhance the rate of arsenic volatilization from soils. Engineered bacteria had been

* Corresponding author. E-mail: gxsun@rcees.ac.cn

reported for bioremediation of other heavy metal (Valls and Lorenzo, 2002; Chen and Wilson, 1997). MerA-mediated mercury detoxification, in which Hg(II) is reduced to volatile Hg(0) (Barkay et al., 2003). Selenium detoxification, in which volatile dimethyl selenide and dimethyl diselenide are formed in bacteria and plants. The success of these strategies are based on the physiological role of these metalloregulatory proteins as specific sensors toward the metals of interest (Bontidean et al., 1998). However, little information about genetic engineered bacteria highly capable of arsenic biovolatilization is available.

The major volatile arsenic compounds formed by microorganisms are arsines, mono-, di-, tri-methylarsine. Microbial biomethylation is one of the major pathway for formation of volatilized arsenic and is now of considerable academic interest (Bentley and Chasteen, 2002). Arsenic biovolatilization is through a reduction of As(V) to As(III) and a series of methylation reactions of As(III) with trimethylarsine (TMA) as a final product (Turpeinen et al., 2002; Bentley and Chasteen, 2002). Recently *Escherichia coli* expressing arsenite S-adenosylmethionine methyltransferase gene (*arsM*) coloned from *R. palustris* was vertified to be able to methylate inorganic arsenic to volatile trimethylarsine (Qin et al., 2006; Yuan et al., 2008; Xu and Rosen, 1997). The practical application of genetic engineering microorganisms for arsenic biovolatilization from arsenic contaminated soils is becoming possible.

To evaluate the possible application of genetic engineering bacteria for bioremediation of arsenic contaminated substrates, the main objective of this work was to express *arsM* gene in isolated arsenic resistant bacteria, to assess biovolatilization of arsenic by engineered bacteria in aquatic and soil environment under laboratory conditions.

1 Materials and methods

1.1 Bacterial strains and molecular techniques

Strains *Bacillus idriensis* and *Sphingomonas desiccabilis* were used in all experiments. The strains were isolated from a sand sample obtained from an arsenic-contaminated soil in Beijing, China. The characteristics of the soil were listed as follows: arsenic 50 mg/kg; Fe 32 mg/kg; Cu 37 mg/kg; Zn 56 mg/kg; Pb 19 mg/kg; pH (H_2O) 6.8; organic matter, 9.32 g/kg; alkali-hydrolysable N 37.9 mg/kg; and available phosphorus 14.6 mg/kg. The medium used for isolation was nutrient agar (0.5% peptone, 0.5% NaCl, 0.2% yeast extract, 0.2% beef extract and 1.5% agar, pH 7.0, all *W*/*V*) containing 5% (*W*/*V*) sodium arsenate (Suresh et al., 2004). The two strains were identified at Institute of Microbiology, the Chinese of Academy.

Strain *B. idriensis* and *S. desiccabilis* with high-level *arsM* expression under the T7 promoter, was used for arsenic-resistance experiments. To construct an expression vector for *arsM*, the *arsM* gene was retrieved from plasmid pET28a (kindly donated by Prof. Barry P. Rosen from Wayne State University, USA). Then the pET28*arsM* was transformed into competent cells (*B. idriensis* and *S. desiccabilis)*. Bacteria cells containing expression vectors were grown for 2 days in LB culture medium at 30°C on the shaker (150 r/min). Total RNA of bacteria was isolated by using an EASYspin (Invitrogen) following the manufacturer's protocol. cDNA was synthesized in 20 μL reactions from total RNA after DNase treatment (Invitrogen, USA), using 200 U of MMLV reverse transcriptase (Invitrogen) and oligo-(dT) as a primer (Sambrook et al., 1989). The PCR program for the amplification of *arsM* gene was run with cDNA as follows: 30 sec at 94°C, 35 cycles of 30 sec at 94°C, and 1 min at 70°C, followed by 10 min at 72°C. PCR using the primers *arsM* for 5′-ATGCCCACTGACATGCAAGAC-3′ and *arsM* back 5′-TCACCCGCAGCAGCGCGCCG-3′. The amplified fragment was 852 bp. To identify transgenic bacteria bearing pET28*arsM*, the PCR product was electrophoresed in 1% agarose gels and detected by staining with ethidium bromide. Transgenic bacteria were used for further experiments.

1.2 Resistance to inorganic arsenic

The arsenate resistance phenotype of cells expressing *arsM* genes was determined in bacteria. Cells of wildtype bacteria (bearing pET28a), and bacteria bearing pET28*arsM* were used for inorganic arsenic sensitivity assays, grown aerobically at 37°C in LB medium, cells of wildtype bacteria (bearing pET28a), and bacteria bearing pET28*arsM* were grown overnight in a LB medium at 37°C, supplemented with containing 25 μg/mL kanamycin. Overnight cultures were diluted 100-fold in LB medium containing 0.3×10^{-3} mol/L isopropyl β-d-thiogalactoside (IPTG), 7×10^{-5} mol/L sodium arsenite. The overnight cultures were then diluted to an A_{600} of 0.1 into the same medium. Bacterial growth was monitored by measuring the optical density at 600 nm with UV-Vis spectrophotometer and continued until the cultures reached stationary phase.

1.3 Volatilization of arsenic in culture medium

To investigate the effects of arsenic methylation by wildtype bacteria and transgenic bacteria (expression *arsM*) in LB medium, arsenite ($NaAsO_2$) was added to each vial at 2×10^{-5} mol/L. Eighty vials of LB medium were inoculated with wild or transgenic bacteria for 0, 6, 18, 24 and 48 hr. Each treatment has four replications. Cells were carefully separated from the solutions by centrifugation (3000 r/min). The supernatant was filter through 0.45 μm filters and kept in the dark on ice until analysis. The cell pellet was then air dried to constant weight at room temperature, weighed into 50-mL extraction vessels, and then steeped in 10 mL of 1% HNO_3 and kept overnight. Extraction of arsenic species in cells were performed by heating these tubes at 95°C for 30 min in a microwave accelerated reaction system (CEM Microwave Technology, USA) (Sun et al., 2008). The supernatant were filtered and kept in the fridge at 4°C. The capped 30-mL vials with two 2-cm nitrocellulose membrane filters (Schleicher & Schuell, New Hampshire) in the cap. Volatile arsenic was trapped with 0.15 mL of 6% H_2O_2 impregnate filters and samples were prepared as reported (Qin et al., 2006).

The solution can be obtained by digesting the filters with 0.2 mL of 70% HNO_3 at 70°C for 20 min after the reaction, then it was diluted 20-fold and analyzed by high performance liquid chromatography-inductively coupled plasma mass spectrometry (HPLC-ICP-MS, 7500, Agilent Technologies, USA).

1.4 Volatilization of arsenic from As-contaminated soil

In order to test the arsenic volatilization from As-contaminated soil, the vials containing 10 g sterilized soil were incubated with 3.5 mL of wildtype or transgenic bacteria. The soil used was an As-contaminated soil (42.36 mg/kg) collected in Beijing, China. Incubation lasted 30 days. Every 5 days four vials containing soil were sequentially extracted with 0.10 mol/L H_3PO_4 and 0.10 mol/L NaOH by shaking for 24 hr (Khalid et al., 2009). The solution was filtered through 0.45 μm filters and kept in the dark on ice for arsenic species analysis. The capped 30 mL vials with two 2 cm nitrocellulose membrane filters in the cap. Every two days open the cap, to replace nitrocellulose membrane filters meanwhile make oxygen into treatment. Nitrocellulose membrane filters captured violatile arsenic, and then the filters were digested with 0.2 mL of 70% HNO_3 at 70°C for 20 min, which was diluted 20-fold.

1.5 Determination of arsenic species and total arsenic

The concentrations of total arsenic were measured by ICP-MS (Qin et al., 2006). Arsenic speciation in the LB medium and soil extracts were determined by HPLC-ICP-MS (Wang et al., 2008). Briefly, the anion-exchange column of PRP-X100 10 μm (250 × 4.6 mm) with a precolumn (11.2 mm) was used for chromatographic separation. The mobile phase consisted of 6.66 mmol/L ammonium hydrophoshpate ($NH_4H_2PO_4$) and 6.66×10^{-3} mmol/L ammonium nitrate (NH_4NO_3). The solution was adjusted to pH 6.2 using ammonia. Retention time for the arsenic species was determined using a species mix comprising stands of 10 μg/L arsenite, arsenate, DMA and MMA.

2 Results

2.1 Identification of isolated bacteria and expression of *arsM* in bacteria

The two strains which exhibited relatively high arsenic resistance were isolated from As-contaminated soil. One isolated strain (*B. idriensis*) was Gram-positive, and has the the ability to form endospores. Colonies are white pigmented and rod-shaped, 1.3–2.1 μm length and 0.3–0.5 μm wide. The colonies was cream, circular, raised, smooth, convex on nutrient agar. Another isolated strain (*S. desiccabilis*) was Gram-negative, colonies are yellow-pigmented non-motile, small rods of 0.21–0.36 μm in diameter and 0.4–0.5 μm in length, and extremely mucoid, convex, round and smooth. Both of them exhibited maximum similarity (99%) at the 16S rRNA gene level with *B. idriensis* and *S. desiccabilis*. On the basis of these characteristics, the isolates were identified as *B. idriensis* and *S. desiccabilis*. The *arsM* gene from *R. palustris* was successfully expressed in *S. desiccabilis* and *B. idriensis* strains which do not have *arsM* gene (Fig. 1). Expressions of *arsM* in bacteria were demonstrated by detection of a strong band corresponding to the expected size of *arsM* gene (Fig. 1).

2.2 Increased arsenic resistance of transgenic bacteria

Cells of *B. idriensis* and *S. desiccabilis* strain, expressing *arsM* gene were used. Both strains with empty pET-28a vector plasmid were used as the negative control. The ability of the engineered cells to resist arsenic were investigated by adding 7×10^{-5} mol/L sodium arsenite to the growth medium. Expression of *arsM* allowed for growth in concentrations of As(III) as high as 7×10^{-5} mol/L after more than a day of incubation. Expression of *arsM* conferred much higher resistance than that of the wild type (Fig. 2). The growth curve of the formers always preceded the wild type. For *S. desiccabilis*, the cell density of expression *arsM* gene in *S. desiccabilis* was 2 times higher than corresponding negative control (Fig. 2).

2.3 Volatilization of arsenic from culture solution

When cells were incubated with As(III) (2×10^{-5} mol/L) for some extended periods, the total amount of arsenic in the culture decreased. The reaction was repeated in a closed vial, and gaseous products were trapped on filters saturated with 6% H_2O_2 and analyzed for arsenic by ICP-MS. Two *arsM* expressed bacteria showed analogous effect (Fig. 3). The amount of volatilized arsenic correlated with the disappearance of total arsenic in solution (Fig. 3). The decrease in As(III) was correlated with the appearance of two soluble products in the medium, DMA(V), As(V), and arsenic in the head space of the reaction vial (Table 1). In the aquatic systems, strains expressing *arsM* in *S. desiccabilis* and *B. idriensis* methylated about 10-fold higher levels of gaseous arsenic respectively than the wild type cells without expression of *arsM* (Table 1). These results indicated that the formation of methylated arsenicals is responsible for detoxification. The maximum percentage

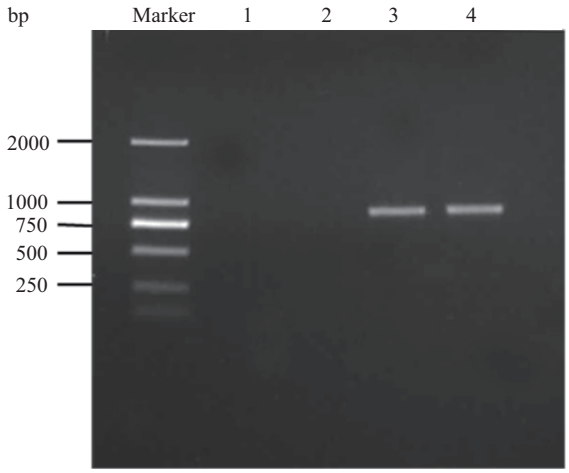

Fig. 1 Amplification of *arsM* by RT-PCR. Samples were detected for *arsM* gene, vector plasmid pET28a+ (lane 1) and *arsM* gene (lane 3) was overexpressed in *B. idriensis*; vector plasmid pET28a+ (lane 2) and *arsM* gene (lane 4) was overexpressed in *S. desiccabilis*.

Table 1 Arsenic species content in the cell and LB medium and gaseous As*

Treatment	As(III) (mg)	As(V) (mg)	DMA(V) (mg)	Volatile As (mg)	Sum of As (mg)	Vol. As/Sum As (%)
Control	14.89 ± 1.58	ND	ND	ND	14.89	–
S + pET28a	13.23 ± 1.63	0.29 ± 0.06	0.90 ± 0.24	0.57 ± 0.12	15.56	3.66
B + pET28a	12.65 ± 1.75	0.28 ± 0.08	1.45 ± 0.10	0.60 ± 0.13	14.93	4.02
S + pET28*arsM*	7.41 ± 1.12	0.27 ± 0.13	1.71 ± 0.55	5.65 ± 0.71	15.04	37.57
B + pET28*arsM*	6.88 ± 1.81	0.28 ± 0.23	1.56 ± 0.58	6.28 ± 0.77	15	41.87

ND: not detected.
* Bacteria were grown in the LB medium containing 2×10^{-5} mol/L As(III) for 18 hr.

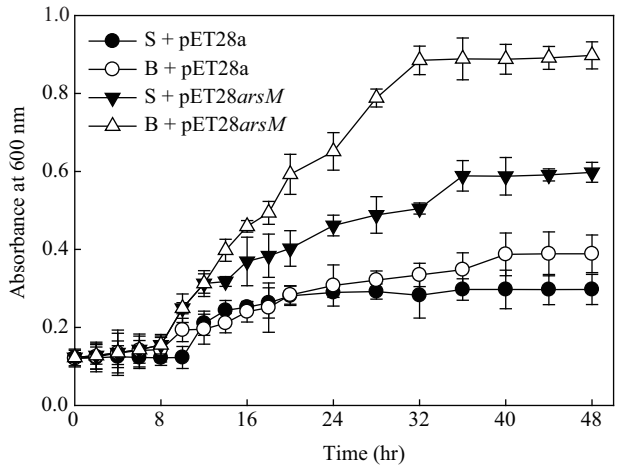

Fig. 2 Growth curve of bacteria with and without *arsM* gene expression in addition of 7×10^{-5} mol/L As(III) in culture medium. The pET28a+ and pET28*arsM*+ represent the strain vector plasmid pET28a and *arsM* gene was overexpressed in *B. idriensis* (B) and *S. desiccabilis* (S). Data shown are the mean values (± standard deviations) from four replicates.

of organic arsenic species in *B. idriensis* strains with the expression *arsM* gene was 49% (Fig. 3a). Although the production of volatile arsenic in *S. desiccabilis* was lower than that of *B. idriensis*, the level of As(III) methylation were very similar to those two corresponding bacteria of expressing *arsM* (Fig. 3b). The increasing amount of the organic arsenic is due to the expression of *arsM* gene, which enhanced the arsenic methylation.

2.4 Volatile removal of arsenic by genetic engineering bacteria from contaminated soil

To investigate this possibility of recombinant cells for arsenic treatment in the soil, bacteria were incubated in As-contaminated soil (42.36 mg/kg arsenic), and a time course experiment was conducted. Arsenic speciation was determined after an incubation of 2 days. No organic arsenic species were detected in the control treatment in which soil was sterilizated with LB addition. The amount of organic arsenic species, i.e., DMA and MMA, accumulated in the medium of the other treatments, and increased with a concomitant increase in gaseous arsenic. The amount of organic arsenic species in *B. idriensis* strains expressing *arsM* gene was higher than the other treatments (Fig. 4). At the end of this experiment (day 30), the decrease of arsenic was related to the appearance of three soluble products in the medium, As(III), DMA, MMA, and gaseous arsenic in the head space of the reaction vial. In soil system, about 2.2%–4.5% of arsenic was removed by biovolatilization during 30 days (Table 2). The ratio of organic arsenic (including gaseous arsenic) (13.87%) is the largest in *B.*

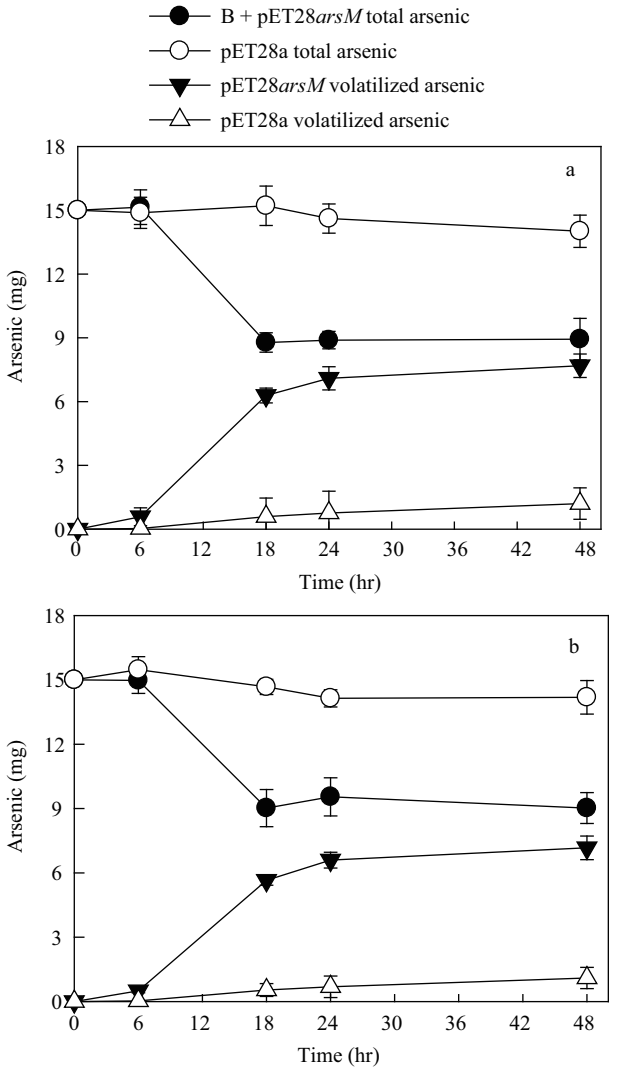

Fig. 3 Sum of As speciation detected from cells and filtrated LB medium and volatilized As (trapped in the filter paper). The pET28a+ and pET28*arsM*+ represent the strain containing vector plasmid pET28a and *arsM* gene overexpressed in *B. idriensis* (B) (a) and *S. desiccabilis* (b). They were grown in 10 mL of LB medium in the presence of 2×10^{-5} mol/L sodium arsenite (S). Data shown are the mean values (± standard deviations) from four independent assays.

idriensis strains expressing *arsM* gene. The high amount of volatile arsenic suggested that these bacteria may be suitable for arsenic removal in contaminated soil.

3 Discussion

In recent years, enhanced biosorbents have been engineered by genetic incorporation of metal-binding peptides such as metallothioneins (Sousa et al., 1998) or synthetic

Table 2 Bacteria were grown in soil (42.36 mg/kg As) for 30 days

Treatment	As(III) (mg/kg)	As(V) (mg/kg)	DMA (mg/kg)	MMA (mg/kg)	Volatile As (mg/kg)	Sum of As (mg/kg)	Vol. As/Sum As (%)
Control	1.97 ± 0.06	33.84 ± 0.59	0.13 ± 0.00	0.11 ± 0.00	0.12 ± 0.00	36.17	0.33
S + pET28a	2.79 ± 0.06	31.07 ± 0.54	0.72 ± 0.00	0.70 ± 0.01	0.78 ± 0.00	36.06	2.2
B + pET28a	2.91 ± 0.08	30.11 ± 0.32	0.92 ± 0.01	1.00 ± 0.01	1.01 ± 0.01	35.95	2.8
S + pET28*arsM*	2.99 ± 0.03	29.02 ± 0.54	1.32 ± 0.00	1.34 ± 0.05	1.44 ± 0.02	36.11	3.9
B + pET28*arsM*	2.92 ± 0.04	28.15 ± 0.28	1.84 ± 0.02	1.84 ± 0.03	1.65 ± 0.01	36.40	4.5

* The content of organic As accumulation consisted of As speciation detected from treatment soil extraction.
The *arsM* gene was overexpressed in genetic strains.

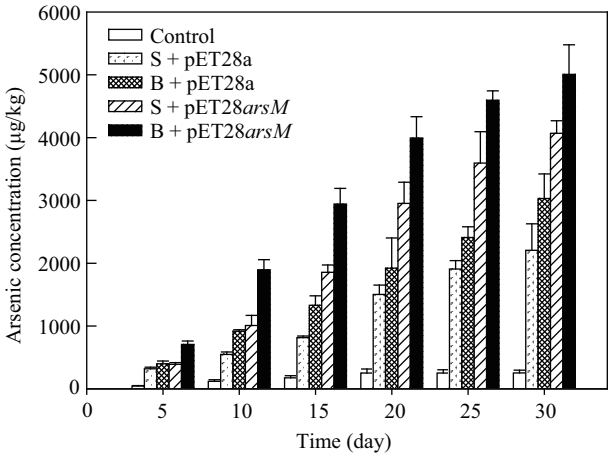

Fig. 4 Sum of organic As in the soil and volatile As. The As concentration in the soil was 42.36 mg/kg. Data shown are the mean values (± standard deviations) from four independent assays.

phytochelatins in different eukaryotes, bacteria (Bae et al., 2000, 2001). Some studies confirmed that multiple physiological responses are induced by arsenic in resistant bacteria, and not exclusively associated with the expression of genes of classical arsenic resistance operons. The expression of such proteins could potentially be used in the construction of recombinant bacteria for the bioremediation of As-contaminated soil and waters. In this article, the expression of *arsM* in indigenous bacteria is proved to be effective for arsenic removal from contaminated soil and water by biovolatilization. In the aquatic systems, strains expressing *arsM* in *S. desiccabilis* and *B. idriensis* methylated about 10-fold higher levels of gaseous arsenic respectively than the corresponding wildtype cells without expression of *arsM*. Previously, some arsenic specific regulatory proteins were over-expressed in *Escherichia coli* cells, and specific arsenic accumulation was achieved in *E. coli* cells. The *arsM* gene from *R. palustris* conferred arsenite resistance to *E. coli* and catalysed trimethylarsine formation from arsenite (Qin et al., 2006; Yuan et al., 2008). An engineered *E. coli* strain expressing the arsenic binding metallothionein of *Fucus vesiculosus* upgraded the concept of using metallothioneins for arsenic removal (Merrifield et al., 2004). The *F. vesiculosus* metallothionein binding to arsenic was co-overexpressed with an arsenic transporter GlpF for removal of arsenic. The engineered *E. coli* can be used to completely remove 35 μg/kg of As(III) in 20 min (Singh et al., 2008). Overexpressing the arsenic-specific regulatory protein ArsR in *E. coli* increased the cellular concentration of arsenic, the resulting strain was able to accumulate up to 50 μg/kg of arsenite (100% removal) from arsenic contaminated water (Kostal et al., 2004). The major volatile arsenic should be TMA due to the overexpression of *arsM* gene in bacteria. The toxicity of TMA is very low, having LD_{50} of 20,000 mg/L (Cullen and Bentley, 2005). Considering low toxicity and concentration of TMA in the air, it could not cause health problem.

The present study demonstrated the potential of arsenic removal from As-contaminated soil via biovolatilization by expressing *arsM* in indigenous bacteria. Some data showed that it was a detoxifying process via the methylation of environmental arsenic by conversion to soluble and gaseous methylated species. This may contribute to global cycling of arsenic. In addition, a number of microorganisms have been shown to methylate arsenic giving a rise to mono-methyl, dimethyl, and/or tri-methyl derivatives. The expression of *arsM* gene in indigenous bacteria will greatly methylate As(III) to the relatively less toxic pentavalent species DMA(V) and TMAO (Yuan et al., 2008). The more toxic MMA(III) and DMA(III) are probably transient intermediates that do not accumulate. Even though the final product is trivalent TMA(III), its volatilization decreased the arsenic concentration in the medium, augmenting detoxification (Yuan et al., 2008). It has been reported that the use of engineered microorganism as binding ligands enhanced the removal of heavy metal ions from contaminated water (Bontidean et al., 1998). Arsenic detoxification genes have been utilized in designing potential arsenic remediation strategies (Dhankher et al., 2002). The results showed that the expression of *arsM* in indigenous bacteria systems increased the arsenic methylation ability not only in aqueous environment but also in arsenic-contaminated soil.

4 Conclusions

In summary, the expression of *arsM* in bacteria significantly increased the ability of bacteria to methylate arsenic in aqueous and soil systems. In the aqueous systems, compared with the wild type cells without the expression of *arsM*, about 10-fold higher levels of gaseous arsenic was generated by *S. desiccabilis* and *B. idriensis* strains with the expression of *arsM*, and biovolatilization by these strains can result in about 2.2%–4.5% of arsenic removal from the soil after 30-day incubation. It is proposed that genetically engineered bacteria with *arsM* could be a promising strategy for bioremediation processes of arsenic-contaminated environments.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 40973058) and the Ministry of Science and Technology (No. 2007CB407304).

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