Functions and unique diversity of genes and microorganisms involved in arsenic methylation in the arsenic-rich shallow and deep sediments of Jiangnan Plain

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ABSTRACT: Almost nothing is known about the activities and diversities of microbial communities involved in As methylation in arsenic-rich shallow and deep sediments; the correlations between As biomethylation and environmental parameters also remain to be elucidated. To address these issues, we collected 9 arsenic-rich sediment samples from the depths of 1, 30, 65, 95, 114, 135, 175, 200 and 223 m in Jiangnan Plain. Microcosm assay indicated that the microbial communities in all of the sediment samples significantly catalyzed arsenic methylation. We identified 90 unique *arsM* genes from the eight samples, all of which code for new or new-type ArsMs, suggesting that As-methylating microorganisms are widely distributed in the samples from shallow to deep sediments. To determine whether biomethylation of As occurs in the sediments under natural geochemical conditions, we conducted microcosm assays without exogenous As and carbons. After 80.0 days of incubation, approximately $4.5\text{--}15.5\ \mu\text{g L}^{-1}$ DMAsV were detected in all of the microcosms with the exception of that from 30.0 m, and $2.0\text{--}9.0\ \mu\text{g L}^{-1}$ MMAsV were detected in the microcosms of 65, 135, 200, and 230 m; moreover, approximately $36.4\text{--}97.2\ \mu\text{g L}^{-1}$ soluble As(V) were detected from the nine sediment samples. This suggests that approximately 5.3%, 0%, 8.1%, 28.9%, 18.0%, 8.7%, 13.8%, 10.2% and 14.9% of total dissolved As were methylated by the microbial communities in the sediment samples from 1, 30, 65, 95, 114, 135, 175, 200 and 223 m without exogenous As and carbons, respectively. The concentrations of biogenic DMAsV show significant positive correlations with the depths of sediments, and negative correlations with the environmental NH_4^+ and NaCl, but show no significant correlations with other environmental parameters, including NO_3^- , SO_4^{2-} , TOC, TON, Fe, Sb, Cu, K, Ca, Mg, Mn and Al. This work for the first time revealed the activities and diversities of As-methylating microbes in the arsenic-rich shallow and deep sediments, and helps to better understand the microorganisms-mediated biogeochemical cycles of arsenic in arsenic-rich shallow and deep sediments.

1 INTRODUCTION

Arsenic (As) is widely distributed in the Earth's crust at an average concentration of $2.0\ \text{mg kg}^{-1}$. It occurs in more than 200 minerals, usually in combination with sulfur and metals (Nordstrom, 2002). Arsenic-contaminated groundwater exists in more than 70 countries worldwide, including Bangladesh, India, Pakistan, Burma, Nepal, Vietnam, Cambodia, China and United States (Kirk *et al.*, 2004; Gu *et al.*, 2012; Shao *et al.*, 2016). Natural processes, biochemical reactions and anthropogenic activities are responsible for the dissolution and release of arsenic from minerals into groundwater (Fendorf *et al.*, 2010). Many bacteria, archaea, fungi, and animals are able to methylate As (Zhu *et al.*, 2014). Arsenic methylation was catalyzed by As(III)-S-adenosylmethionine methyltransferase (ArsM) (Ajees *et al.*, 2012). The first *arsM* gene was cloned from the soil bacterium *Rhodopseudomonas palustris* (Qin *et al.*, 2006). It codes for a protein with 283 amino acid residues. ArsM is present

in both prokaryotic and eukaryotic microorganisms (Zhu *et al.*, 2017). Purified ArsM can convert As(III) into DMAsV, TMAO, and volatile TMAsIII (Chen *et al.*, 2014; Jia *et al.*, 2013; Slyemi *et al.*, 2012; Zhu *et al.*, 2014). The *arsM* gene can be used as a molecular marker for investigations of the diversities of As(III)-methylating microbes (Qin *et al.*, 2006, 2009). Recently, some single bacterial strains with significant As(III)-methylating activities were isolated from paddy soils, wastewater ponds, and microbial mats (Huang *et al.*, 2016; Kuramata *et al.*, 2015; Wang *et al.*, 2016; Zhang *et al.*, 2015b). Functional analyses suggest that ArsMs play a role in the detoxification of As(III) and could be exploited in bioremediation of arsenic-contaminated groundwater (Huang *et al.*, 2015; Saunders *et al.*, 2016).

This study aimed to explore the microbial communities-catalyzed methylation of arsenic in the arsenic-rich sediments in Jiangnan Plain. To the best of our knowledge, this is the first effort to characterize the functions and diversities of microbial communities

involved in arsenic methylation, and the correlations between arsenic biomethylation and environmental parameters in the severely contaminated shallow and deep sediments. This work gained a new understanding of the microorganisms-mediated biogeochemical cycles of arsenic in arsenic-rich sediments.

2 METHODS/EXPERIMENTAL

2.1 Sampling

The sampling site (113°36'35.028"E, 30°8'34.944"N) was near a paddy field, located in the Jiahe village that is affiliated to the Shahu town of Xiantao city, Hubei province, China (Fig. 1a). Sediment samples were collected from the depths of 1, 30, 65, 95, 114, 135, 175, 200, and 223 m. The external layers of the sediment cores were carefully removed to avoid contaminations and oxidation by oxygen. The samples were placed into sterilized tubes, and kept on ice immediately. All of the samples were transported into the laboratory in 12.0 hours.

2.2 Geochemical analyses

To determine the total arsenic content, one gram of dried sample powders was mixed with 20.0 mL of aqua regia. After 2.0 hours of incubation at 100°C, the mixture was centrifuged, and the supernatant was collected for determination of the arsenic concentration using the atomic fluorescence spectrometry (AFS) (AFS-9600, Haiguang, Beijing, China). Soluble arsenic species, including As(III), As(V), MMAs^V and DMAs^V, were determined by high-performance liquid chromatography linked to atomic fluorescence spectrometry (HPLC-ICP-MS) (LC-20A, Shimadzu, Japan; ELAN, DRC-e, PerkinElmer, USA).

2.3 Determination of As-methylating activities of the sediment samples

To detect the As-methylating activity of the microbial community from sediment samples, active microcosms were prepared in triplicate by inoculating 8.0 g of samples into 20.0 mL of simulated groundwater (Wang et al., 2017) amended with 0.23 mM As(III) and 20.0 mM lactate in a 50-mL flask. All of the mixtures were incubated at 30°C without shaking. After 80 days, approximately 1.0 mL of cultures was removed from each flask for determination of the concentrations of MMAs^V and DMAs^V using HPLC-ICP-MS.

2.4 Amplification, cloning and analysis of *arsM* genes from genomic DNAs of the samples

To detect the diversity of As-methylating microbes from the samples, three pairs of primers were used to amplify *arsM* genes from the genomic DNA of each sample. The primers were listed in Table 1. Metagenomic DNA was extracted from the samples using the MiniBEST bacterial genomic DNA extraction kit

(TaKaRa, Japan) (Plenge et al., 2016). PCR products were gel purified using the E.Z.N.A gel extraction kit (Omega, USA). Purified DNA was ligated into a T vector. An *arsM* gene library was generated by introducing the recombinant plasmids into *Escherichia coli* DH5 α competent cells. All of the clones from the library were sequenced and analyzed as described previously (Zhong et al., 2017).

2.5 Methylated As release assay in the absence of exogenous As and carbons

To determine whether microbial communities from the sediment samples catalyze arsenic methylation under natural conditions, we prepared microcosms without addition of exogenous As and carbon sources. Eight grams of the sediment samples were mixed with 20.0 mL of simulated groundwater in a 50-mL flask. All of the microcosms were incubated at 30°C for 80.0 days without shaking. After 80.0 days of incubation, approximately 1.0 mL of slurries was removed from the flasks for measurement of the concentrations of As(III), As(V), MMAs^V and DMAs^V.

2.6 Analysis of the correlations between methylated As and geochemical parameters

The Spearman's Rank Correlation Coefficient was used to determine the correlations between the concentrations of methylated As species produced by microbial communities, and the geochemical parameters in the environment. The SPSS software was used for statistical analyses. Correlations were considered to be statistically significant at 95% confidence level ($P < 0.05$).

3 RESULTS AND DISCUSSION

3.1 Characterization of the sampling site

We collected nine sediment samples from the depths of 1, 30, 65, 95, 114, 135, 175, 200, and 223 m, respectively. Figure 1b shows the appearances of the sediment samples. Geochemical analyses indicated that the sediments 180 contain high contents of total As (ranging from 6.74 to 27.9 mg/kg) and soluble As (ranging from 1.9 to 100.7 μ g/L). The arsenic contents showed no correlations with the depths of the sediments. The concentrations of total arsenic also showed no correlations with those of soluble arsenic; this suggests that multiple factors controlled the arsenic dissolution and release from the sediments. The sediment samples contain 0.27–8.37 g/kg total organic carbons (TOC) and 0.18–1.26 g/kg total organic nitrogen (TON); these substances could provide essential carbon and nitrogen sources for the growth of microorganisms in the sediments. The sediments also contain relatively high contents of sulfate (ranging from 14.53 to 863.87 mg/kg), and relatively low contents of ammonium (ranging

Figure 1. Illustrations of the sampling site and sample appearances. (a) Geographical map of the sampling location in the Jiahe village of the Shahu town in Jiangnan Plain. (b) The appearances of the sediment samples collected from the depths of 1 to 230 m.

from 3.12 to 52.77 mg/kg) and nitrate (ranging from 0.23 to 3.03 mg/kg).

3.2 *As-methylating activities of the microbial communities from the sediments*

Microcosm assay was used to determine the As-methylating activities of the nine sediment samples. The microcosms were amended with 0.23 mM As(III) as the substrates of ArsM enzymes, and 20.0 mM lactate as the carbon sources. The results showed that no detectable amounts of methylated arsenic species were observed in the autoclaved sediment slurries. In contrast, when the sediment microcosms were not autoclaved, approximately 2.06, 35.53, 18.83, 53.99, 121.86, 7.54, 7.00, 260.38, and 3.02 $\mu\text{g/L}$ DMAs^V were detected in the microcosms from 1, 30, 65, 95, 114, 135, 175, 200, and 223 m, respectively (Figs. 2a and 2b). No significant amounts of MMAs^V were detectable in all of the sediment samples with the exception of that from the depth of 135 m, in which 3.30 $\mu\text{g/L}$ of biogenic MMAs^V was produced. This suggests that the microbial communities in the nine sediment samples were able to significantly catalyze As methylation, and the dominant products were DMAs^V.

3.3 *Unique diversities of As-methylating microbes in the sediment samples*

To understand the microbial basis of the As-methylating activities of the nine sediment samples, we explored the diversities of the As-methylating microbes present in the microbial communities by cloning, sequencing and analyzing the *arsM* genes from the metagenomic DNAs of the samples. The results showed that we identified 90 different ArsM proteins from the microbial communities of the eight samples, including 20, 22, 8, 10, 4, 5, 13, and 7 different ArsMs from the samples of 1, 30, 65, 95, 114, 135, 200, and 223 m, respectively (Fig. 3a). A phylogenetic tree was constructed based on the multiple

Figure 2. Arsenic methylation activities of the microbial communities of the nine samples collected from the arsenic-rich shallow and deep sediments in Jiangnan Plain. (a) HPLC-ICP-MS chromatograms of the methylated As species produced by the microbial communities of the samples from 1 to 223 m after 80.0 days of incubation. Curve 1, standards of DMAs^V and MMAs^V; curve 2, DMAs^V and MMAs^V concentrations in the microcosms amended with 0.23 mM As(III) as the substrates of ArsM enzymes, and 20.0 mM lactate as the carbon sources after 80.0 days of incubation. (b) Concentrations of the methylated As in the microcosms of the samples from 1 to 223 m after 80.0 days of incubation.

alignments of the ArsM proteins from this study and their closely related ArsMs from other known microorganisms (Fig. 3b). An ArsM sequence from archaea was chosen as the outgroup.

3.4 *Methylated As release from the sediments*

To detect whether the microbial communities-catalyzed As methylation occurs under natural conditions, we performed methylated As release assay using the microcosms in the absence of any exogenous organic carbons and As(III) compounds. We found that approximately 5.0, 30.0, 55.5, 126.1, 5.7, 25.2, 260.3, 9.0 $\mu\text{g/L}$ DMAs^V were produced in the slurries of the samples from the depths 30, 65, 95, 114, 135, 175, 200, and 223 m, respectively, and no significant amount of DMAs^V were detected from the microcosm of 30 m (Fig. 4a). We also examined the concentrations of soluble As in the sediment samples after 80.0 days of incubation. The results showed that approximately 85.05, 18.69, 86.64, 36.36, 39, 97.2, 50.79, 151.53 and 84.09 $\mu\text{g/L}$ of soluble As(V) were detected in the slurries of the samples from 1, 30, 65, 95, 114, 135, 175, 200 and 223 m, respectively (Fig. 4b). This suggests that approximately 5.29%, 0%, 8.08%, 28.87%, 17.95%, 8.74%, 13.78%, 10.23% and 14.87% of total

Figure 3. Characterization of the ArsM proteins identified from microbial communities of the sediment samples from Jiangnan Plain. (a) The numbers of different ArsM proteins identified from the microbial community of each sample from the depths of 1 to 223 m. (b) Maximum likelihood tree constructed from the multiple sequence alignments of the obtained ArsM proteins from the sediment samples and their closely related homologues from other known microorganisms. All of the ArsM sequences in this study are shown in red; numbers on the branches are bootstrap values based on 1000 replicates; only the bootstrap values larger than 50% are shown; the scale bar represents the average number of substitutions per site.

soluble As were methylated by the microbial communities in the microcosms of the samples from 1, 30, 65, 95, 114, 135, 175, 200 and 223 m, respectively.

3.5 Correlations of the methylated As species and some geochemical parameters

The Spearman's Rank Correlation Coefficient was used to discover the correlations between methylated As species and geochemical parameters. We found that the concentrations of biogenic DMAs^V from the sediments of different depths show significant positive correlations with the depths of the sediments

Figure 4. The microbial communities-catalyzed methylation and dissolution of arsenic in the microcosms prepared from the sediment samples of the depths 1 to 223 m in the absence of exogenous As and carbons. (a) Concentrations of biogenic methylated As after 80.0 days of incubation. (b) Concentrations of biogenic soluble As after 80.0 days of incubation.

Figure 5. Correlations between arsenic biomethylation and some environmental parameters. (a) Correlation between biogenic DMAs^V and sediment depths ($r = 0.78565$, $p = 0.01209$); (b) Correlation between biogenic DMAs^V and concentrations of environmental NH_4^+ ($r = -0.60782$, $p = 0.0825$); (c) Correlation between biogenic DMAs^V and concentrations of environmental Na^+ ($r = -0.78529$, $p = 0.01215$); (d) Correlation between biogenic DMAs^V and concentrations of environmental Cl^- ($r = -0.67712$, $p = 0.04512$).

($r = 0.78565$; $p = 0.01209$) (Fig. 5a), and negative correlations with the contents of NH_4^+ ($r = -0.60782$; $p = 0.0825$), Na^+ ($r = -0.78529$; $p = 0.01215$), and Cl^- ($r = -0.67712$; $p = 0.04512$) in the environment (Fig. 5b, 5c and 5d).

4 CONCLUSIONS

This study aimed to explore how arsenic was released from soil phase into groundwater, and how sulfate affects this bioprocess in Jiangnan Plain, China. We found that a large diversity of DARPs are present in the shallow soils from the depths of 1.6 and 4.6 m. Microcosm assays indicated that the microbial communities significantly catalyzed the dissolution, reduction and release of arsenic and iron from the soils into aqueous phase. It is interesting to see that addition of 10.0 mM sulfate into the microcosms led to a significant increase of the microorganisms-mediated release of arsenic and iron from the soils. Quantitative PCR analysis for the functional gene abundances suggested that the sulfate-induced increase of microbial release of arsenic and iron was attributed to the significant enhancement of the DARP growth by sulfate. These results suggested that agricultural uses of sulfate fertilizers enhanced arsenic contamination in groundwater. The findings of this study gained new insight into the mechanisms by which the arsenic-contaminated groundwater was formed, and gave direct evidence that agricultural activities may enhance the arsenic contamination in groundwater.

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