Metagenomic analysis revealed highly diverse microbial arsenic metabolism genes in paddy soils with low-arsenic contents

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A B S T R A C T

Microbe-mediated arsenic (As) metabolism plays a critical role in global As cycle, and As metabolism involves different types of genes encoding proteins facilitating its biotransformation and transportation processes. Here, we used metagenomic analysis based on high-throughput sequencing and constructed As metabolism protein databases to analyze As metabolism genes in five paddy soils with low-As contents. The results showed that highly diverse As metabolism genes were present in these paddy soils, with varied abundances and distribution for different types and subtypes of these genes. Arsenate reduction genes (ars) dominated in all soil samples, and significant correlation existed between the abundance of arr (arsenite respiration), aio (arsenite oxidation), and arsM (arsenite methylation) genes, indicating the co-existence and close-relation of different As resistance systems of microbes in wetland environments similar to these paddy soils after long-term evolution. Among all soil parameters, pH was an important factor controlling the distribution of As metabolism gene in five paddy soils (p = 0.018). To the best of our knowledge, this is the first study using high-throughput sequencing and metagenomics approach in characterizing As metabolism genes in the five paddy soil, showing their great potential in As biotransformation, and therefore in mitigating arsenic risk to humans.

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1. Introduction

Arsenic (As) is a notoriously poisonous trace element which is ubiquitous in natural environment and can enter into the food chain through various pathways (Cai et al., 2013; Xiao et al., 2012; Zhang et al., 2015; Zhu et al., 2014). Typically, rice can accumulate As from both polluted and unpolluted paddy soils, posing a potential health risk to populations with high rice consumption in China, Southern Asian and certain Latin American areas (Li et al., 2011; Zhu et al., 2014). Microbial mediated As metabolic processes play a major role in As cycling in the plant-soil-microbe system, including arsenite (As(III)) oxidation, arsenate (As(V)) respiration, As(V) reduction, and As(III) methylation (Lett et al., 2012). Recently, anaerobic As(III) oxidation was found in the halooalkaliphilic bacterium *Alkalilimnicola ehrlichii* strain MLHE-1 and the photosynthetic purple sulfur bacterium *Ectothiorhodospira* sp. strain PHS-1 (Kulp et al., 2008; Zargar et al., 2010), which was suggested to be conducted by a new arsenite oxidase clade, ArxA (Zargar et al., 2012), though no information is presently known on the this enzyme’s functioning (van Lis et al., 2013). Through these processes, As speciation and bioavailability are altered, affecting its toxicity, environmental behavior and fate in the environment, and also its accumulation into food crops (such as rice) (Jia et al., 2014, 2013; Stolz and Oremland, 1999).

Arsenic metabolism genes mainly consist of four types: *aio* genes (As(III) oxidation, previously named as *aox/aso/aro*) (Lett et al., 2012), *arr* genes (As(V) respiration) (Malasarn et al., 2004),...
ars genes (As(V) reduction related) (Oremland and Stolz, 2005), and arsM genes (As(III) methylation) (Mestrot et al., 2011; Qin et al., 2006). These genes, and their corresponding proteins or their subunits (subtypes) are frequently used as molecular markers for microbial ecology studies (Lever, 2013; Malasarn et al., 2004; Silver and Phung, 2005). Traditionally, degenerate primer sets were designed and used for many PCR-based methods to investigate the distribution and diversity of one or several subtypes of As metabolism genes (Jia et al., 2014, 2013; Schuchmann and Muller, 2014; Silver and Phung, 2005). Jia et al. (2013) for the first time designed specific primers and successfully amplified prokaryotic arsM gene in 14 tested soils with wide range of As concentrations, and found that microbes containing arsM genes were phylogenetically diverse and widespread. Moreover, their results based on aioA, arsC and arrA highlighted that As uptake by rice is influenced by microbe-mediated As redox processes, which are influenced by root oxygen release and organic matter application (Jia et al., 2014). These techniques are classical but limited due to the bias of the primers and potential nonspecific amplification as well as a lack of available primers sometimes. With the development of high-throughput sequencing techniques and dramatic drop of sequencing prices, diversity analysis based on metagenome becomes feasible, which can target all As metabolism genes and does not rely on the specificity and coverage of the primers used (Cai et al., 2013).

Most As-related studies targeted high-As contaminated environments, while less attention has been paid on natural environments with low-As levels. This study aimed to investigate the distribution, diversity, and abundance of As metabolism genes in typical paddy soils with low-As contents from Southern China. The findings of this study will provide a novel insight into and a better understanding of microbial mediated As metabolism in low-As soil habitats.

2. Material and methods

2.1. Soil sampling and arsenic analysis

Paddy soil samples were collected from five sites: Leizhou in Guangdong Province (LZ), Jiaxing in Zhejiang Province (JX), Yingtan in Jiangxi Province (YT), Gushi of Taoyuan in Hunan Province (TY-G), and Baodongyu of Taoyuan in Hunan Province (TY-B). Soils from the surface (0–20 cm) were taken and divided for DNA extraction and physiochemical analysis. These soils were distinct from each other in soil type, parent material and some other physiochemical properties, as reported previously (Xiao et al., 2014a, 2014b). Total As contents in the soil were detected using method described previously (Sun et al., 2008; Williams et al., 2007; Zhu et al., 2014). Briefly, after air-dried and sieved (mesh size = 150 μm), soil samples and standards with known As concentrations (1.5–2.5 g) were digested with aqua regia (prepared before use), 60% perchloric acid and 1 M hydrogen chloride successively. Arsenic in the residue was dissolved and diluted by adding Milli-Q water, and measured using ICP-MS (7500a, Agilent Technologies), and the recovery efficiency was 88.87 ± 0.01%.

2.2. DNA extraction and sequencing

DNA was extracted from the soil samples in duplicate using the MoBio PowerSoil kit (MOBIO) according to the manufacturer’s protocol. DNA yields of the 10 samples were between 1.0 and 2.5 μg, as quantified using the Quant-IT PicoGreen dsDNA HS assay kit (Invitrogen). Sequencing was performed using Illumina Hiseq 2000 (Illumina) at Majorbio, Inc., Shanghai, China. Raw reads (101 bp in length) were trimmed to remove low quality reads that contained ambiguous nucleotides or had a quality value lower than 20 (Chen et al., 2013). In total, 750,385,006 clean reads were generated with an average of 75,038,500 reads per sample. Data is available at the NCBI Short Read Archive under accession number SRP039858.

2.3. Bioinformatics analysis

The local BLASTX programs were employed to align trimmed clean reads of each data set against an As metabolism protein database constructed previously (Cai et al., 2013). This database included a total of 17 subdatabases, corresponding to 17 subtypes of As metabolism genes, aio genes (aioX, aioS, aioB, aioA, aioC, and aioD), arr genes (arrA and arrB), ars genes (arsR, arsD, arsG, arsC, arsH and ACR3) and arsM. We used the m 8 output format and e-value cutoffs of 1e⁻³ to do the best BLAST search (Mackelprang et al., 2011). The BLASTX outputs against As metabolism protein database were screened by a self-written script to extract alignments of aligned length ≥25 aa and identity ≥90% (Cai et al., 2009).

![Fig. 1. Total arsenic content (a), composition and abundance of arsenic (As) metabolism genes in these five paddy soils, (b) based on abundances (ppm, one read in one million reads), (c) based on percentages (%). Abbreviation: LZ, Leizhou in Guangdong Province; JX, Jiaxing in Zhejiang Province; YT, Yingtan in Jiangxi Province; TY-G, Gushi of Taoyuan in Hunan Province; TY-B, Baodongyu of Taoyuan in Hunan Province, same for the followings.](image-url)
Based on data from BLAST research and script-screening, the proportions of different types of As metabolism genes were defined as “percentage” (% in “total As metabolism genes sequences”) and “abundance” (ppm, one read in one million reads, in “total metagenome sequences”), respectively (Yang et al., 2013). The MEGA 5 (Tamura et al., 2011) and PAST (Hammer et al., 2001) local tools were used to construct a phylogenetic tree and perform cluster analysis. BAND program (Heinrich-Salmeron et al., 2011) and MATLAB software (http://www.mathworks.com/) were applied to visualize the As metabolism genes abundance profile.

2.4. Statistical analysis

Correlation analyses were carried out using the Pearson correlation method with significance defined at the 0.05 level (SPSS 13.0). Principal component analysis (PCA) was performed based on the abundances of the As metabolism genes subtypes. Additionally, redundancy analysis (RDA) combined with Monte Carlo permutation was conducted to find the relationship between the soil parameters (cf. Table 1 in Xiao et al., 2014a) and the abundance of As metabolism genes using CANOCO 4.5 for Windows (Microcomputer Power) (Xiao et al., 2014a).

3. Results and discussion

3.1. Total arsenic As content in these five soil samples

Total As concentration in all samples except Yingtan (YT) were below the first class standard of environmental quality standards for soils of China (15 mg kg⁻¹, GB 15168-1995), and As concentration in YT was far below the second class standard of Environmental Quality Standards for Soils of China (30 mg kg⁻¹, GB 15168-1995), as shown in Fig. 1a. According to the GB 15168-1995, the first class standard (15 mg kg⁻¹) applies to environments like nature reserve with background As concentration, the second class standard (30 mg kg⁻¹) apply to environments like uncontaminated agricultural lands, indicating that our paddy soil samples can be considered as having low-arsenic level. It was reasonable since all of these samples were taken from rural areas of Southern China, which were generally less exposed to sources like industrial waste or mining drainage (Zhao et al., 2010; Zhu et al., 2014).

3.2. Abundance of As metabolism genes and statistical analysis

The abundance and percentage of As metabolism genes assigned into four types (different metabolism pathways) are shown in Fig. 1b and c. The overall abundance range of As metabolism genes in paddy soil was 80–120 ppm (one read in one million reads), and over 85% of them were As(V) reduction related genes (ars) in all soil samples, which are involved in As detoxification processes, such as cytoplasmic As(V) reduction and As(III) efflux (Silver and Phung, 2005). These genes are also prevalent among sediment and activated sludge (Cai et al., 2013), which are generally characterized by low redox potential. As(III) methylation genes (arsM) constituted secondary important part of total As metabolism genes (6–9%) among all five paddy soils, and higher than aio and arr in our samples, implying a strong potential to produce methylated As species, which could be uptake by rice roots and explain why most rice contains unusually high concentrations.
of methylated As species compared with other cereals (Jia et al., 2013). The low abundance of arr genes compared with the others detected in the five paddy soils, which has also been confirmed either in paddy soils or As and antimony (Sb) contaminated mine field (Jia et al., 2014, 2013; Luo et al., 2014; Zhang et al., 2015), could be partly ascribed to their specific presence in anaerobic As(V) respiring bacteria (Malasarn et al., 2004; Silver and Phung, 2005). Previous studies showed that total As concentrations in paddy soils correlated positively with the abundance of genes involved in As biotransformation processes (Zhang et al., 2015), and concentrations of different As species in soil solutions have also revealed good correlations with the abundance of genes responsible for different As biotransformation processes in microcosm experiments (Jia et al., 2014, 2013). Our results showed no significant correlation between total As content and abundance of different types of As metabolism genes (Table S1), which were possibly due to the low As level in these soil samples.

Redundancy analyses (RDA) of 17 As metabolism genes (Fig. S1) and soil parameters (Xiao et al., 2014a) showed that pH was significantly correlated with gene abundance variation among different paddy soil (p = 0.018), contributing 52% to the variance (Fig. 2), suggesting that microbially-mediated As metabolic processes in paddy soils were sensitive to pH variation, as suggested previously (Stolz and Oremland, 1999). Actually, the pH value is known to be an important factor in many microbial processes, such as acetogenesis, ammonia oxidation, CO₂ fixation, and methanogenesis, and worked mainly by influencing nutrients or substrates availability and microbial cell physiology (Schuchmann and Muller, 2014; Taconi et al., 2008; Xiao et al., 2014a; Zhang et al., 2012). Significant correlation existed between the abundance of arr, aio, and arsM genes, but not arr genes (Fig. 3). Zhang et al. (2015) also revealed the strong correlation of arsC and arsM gene copies based on quantitative PCR, and suggested that similar compositions of microbial communities are involved in As(V) detoxification and As(III) methylation in paddy soil. Considering that arr, aio, and arsM genes are separately involved in cytoplasmic As(V) reduction, As(III) oxidation, and As(III) methylation processes, the significant correlation between the abundances of arr, aio, and arsM gene indicated the co-existence and close-relationship of these different As resistance systems in microbes in paddy soil (Jia et al., 2014, 2013; Zhang et al., 2015; Zhu et al., 2014).

3.3. Distribution, diversity, and abundance of aioA and arrA genes

To investigate the potential As redox in the environment, aioA has been proposed as a valuable functional marker gene responsible for As(III) oxidation and arrA as a reliable marker gene for As(V) respiration reduction (Malasarn et al., 2004; Quéméneur et al., 2008; Silver and Phung, 2005). AioA and ArrA proteins are both molybdenum-containing enzymes in the dimethyl sulfoxide (DMSO) reductase family (Oremland and Stolz, 2005; Silver and Phung, 2005), sharing similar amino acid length and structural characteristic while carrying out completely distinct functions. The diversity, distribution, and abundance of both genes were further mined here. As shown in Fig. 4, they were divided into two clades clearly, displaying remarkable heterogeneous abundance profile.
among the 5 samples, as a few of the *aioA* and *arrA* genes distributed in more than three environments (e.g. *Ralstonia* sp. 22 (ACX69823)) while some specifically existed in only one environment (e.g. *Thiomonas intermedia* K12 (YP_001098817)). Researchers have discovered general consistence between *aioA* and 16S rRNA gene phylogeny in particular field, as has been underlined for other functional marker genes (Inskeep et al., 2007; Jackson and Dugas, 2003; Klein et al., 2001; Wagner et al., 1998). The most abundant *aioA* sequence in all five studied samples was derived from *Nitrobacter hamburgensis* X14 (YP_571843). Among the 26 detected *aioA* sequences, the majority were from Burkholerials and Rhizobials at order level, which were also the two main microbial communities in rhizospheric bacteria and contributed significantly to the oxidation of As(III) in soils under both aerobic and anaerobic conditions (Jia et al., 2014; Rhine et al., 2005). In paddy TY-B, *arrA* genes were detected with the highest diversity but with the least abundance, and the lowest diversity was found in paddies LZ and YT. In addition, the shared dominant *arrA* gene sequences in all 5 samples were derived from Opitutus terraet PB90-1 (YP_001818350). Contrary to *aioA* genes, high diversity (including Firmicutes, Chlamydiae, Proteobacteria, Deferribacteres and Chrysiogenetes) of As(V)-respiring systems in paddy soil appeared in phylogenetic analysis of the *arrA* gene sequences. From an evolutionary perspective, it is intriguing that As(V)-respiring bacteria are phylogenetically diverse while *arrA* gene sequences are well-reserved at the same time (Malasarn et al., 2004), one speculation could be the deceased evolution since the Great Oxygenation Event of Earth, which resulted in increased oxygen concentration in the atmosphere and evolution of aerobic respiration (Schirrmeister et al., 2013).

### 3.4. Distribution, diversity, and abundance of *arsC* genes

The well-studied As(V) reducing pathways typically include detoxification reduction (*ArsC*) and dissipatory respiration (*ArrA/B*). *ArrA/B* is either membrane-bound or free in periplasm, while *ArsC* located in cytoplasm could reduce As(V) within cytoplasmic membrane and subsequently excrete As(III) by ArsAB efflux pump (Oremland and Stolz, 2005). Given the habitat type as paddy soil here, As(V) detoxification functioning under both aerobic and anaerobic conditions (Bhattacharjee and Rosen, 2007; Silver and Phung, 2005) appears to be more widespread compared with As(V) respiration which is limited by anaerobic conditions. As shown in Fig. 1, the abundance of *arsC* genes ranged from 81.11 to 103.82 ppm (one read in one million reads), far more than *arr* genes ranging from 0.64 to 1.30 ppm (one read in one million reads), which was similar to Cai et al. (2013), and could be ascribed to the versatile lifestyle of As(V) reducing bacteria (Silver and Phung, 2005). To gain a deep perspective of As(V) reduction, the critical gene *arsC* in As(V) reduction was explored. Clustering based upon abundance of *arsC* indicated the closest proximity between paddies JX and YT, which was grouped with paddy LZ and further clustered with paddy TY-G., and finally with paddy TY-B (Fig. S2).
abundance profile in Figure S2 revealed obvious inhomogeneous distribution among different gene sequences, with ~80% of total \( \text{arsC} \) gene abundance in the five samples was attributed to top 40 abundant sequences. Therefore, top 40 \( \text{arsC} \) sequences were chosen for following phylogenetic analysis, showing a broad phylogenetic distribution of \( \text{arsC} \) genes (Fig. 5), while 45% and 8% of the top 40 \( \text{arsC} \) genes presented were from \textit{Rhizobiales} and \textit{Burkholerials}—also two major groups in \textit{aioA} phylogeny (Fig. 4), and dominating in rice root rhizospheric bacteria (Jia et al., 2014; Rhine et al., 2005). Moreover, the most abundant \( \text{arsC} \) gene was from \textit{A. f i p i a} sp. 1NLS2 (EFI50814), accounting for 27.11% of total \( \text{arsC} \) gene sequences detected. While the \textit{aioA} tree generally reflects the true 16S rDNA phylogeny, the inconsistence (Fig. 5) between the overall phylogeny of 16S rDNA and \( \text{arsC} \) seemed common (Jackson and Dugas, 2003; Zhang et al., 2015), which might be attributed to the role of multiple-cases of chromosomal-plasmid exchange and following horizontal gene transfer happening simultaneously in microbial evolution (Jackson and Dugas, 2003).

3.5. Distribution, diversity, and abundance of \( \text{arsM} \) genes

The tree in Fig. 6 showed that the \( \text{arsM} \) sequences hosted by indigenous microbial community were branching into diverse phyla, mainly composed of \textit{Proteobacteria}, \textit{Firmicutes}, and \textit{Bacteroidetes}, suggesting the genes encoding \( \text{arsM} \) were widespread in paddy soil (Jia et al., 2013). The highest genotype diversity was observed in paddy LZ, while the lowest was in paddies YT and TY-G. The sequence contributing most to abundance in paddies JX, YT, TY-G, and TY-B was from \textit{Candidatus Solibacter usitatus} Ellin6076 (YP.825656), with the exception of paddy LZ from \textit{Rhodopseudomonas palustris}, which was confirmed to produce volatile trimethylarsine (TMAs) as the final product of As(III) methylation reaction (Mestrot et al., 2011). Compared with other investigated environmental sites of similar As background level (mean 3.65%) (Cai et al., 2013), relative abundance of \( \text{arsM} \) gene (mean 8.07%) was higher here, which could be mainly attributed to the unique biogeochemical conditions in paddy soil, such as oxic/anoxic alternate due to root oxygen release, organic matter input from inside (root) and outside (straw), and those abundant autotrophic sulfate-reducing bacteria as well as methanooarchaea, favoring As mobilization and synergy between different microbial groups (Jia et al., 2013; Zhang et al., 2015). The conversion between the more toxic As(III) and the less toxic As(V) cannot remove As from soils, while As(III) methylation mediated by \( \text{arsM} \) and coding protein and subsequent volatilization is an important pathway for As removal from soils and sediments, indicating a high potential for paddy soil to remove As, which has been verified by both lab and field experiments (Huang et al., 2012; Mestrot et al., 2011).

3.6. Possible evolutionary history affecting the abundance and diversity of As metabolism genes

In all five paddy soils with low-As content, most of the detected genes involved in As metabolic processes were responsible for As detoxification, such as \textit{aio}, \textit{ars}, \textit{arsM} (Bhattacharjee and Rosen, 2007; Silver and Phung, 2005; Zhu et al., 2014), indicating the
prevalence of As resistance in microbes. Since As(III) was the dominant As species due to the reducing conditions in primordial atmosphere, early organisms would firstly evolve detoxification mechanisms to cope with As(III) (Rosen, 2002), which might contribute to the most abundant genes involved in As(III) efflux (arsB and ACR3) detected in the five paddy soils even with low-As level (Fig. S1). The mechanisms to cope with As(V) might have evolved to use existing As(III) efflux systems later when the atmosphere became oxidized (Rosen, 2002), for example, the cytoplasmic reduction of As(V) to As(III) mediated by arsC detected in the soils (Fig. 1) followed by As(III) efflux as the detoxification process. For anaerobic As(V) respiration process mediated by arr genes, in which As(V) is used as the anaerobic terminal electron acceptors, though it is still being debated whether it would occur preceding aerobic oxygen-utilizing respiratory electron transport chains. However, the anaerobic bacteria should arise from prebiotic forms hundreds of millions of years before the oxygen respiration first occurs (Silver and Phung, 2005).

4. Conclusion

Our study provided an overall picture of genes related to As metabolic processes in paddy soil by high-throughput sequencing and metagenomics approach. Arsenic metabolism genes were ubiquitous, abundant, and associated with diverse microbes even in low-As environments, and statistical analysis showed pH as an important factor controlling their distribution in paddy soil. ars dominated in all paddy soil samples, and was well correlated with aio and arsM. These results indicate a combinational effect of evolution and selection on As metabolism in microbes under different environmental conditions.

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Appendix A Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2015.12.023.

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