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Arsenic-methylating microbial community in sediment along the water flow is correlated with the distance to a low-temperature hot spring

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ABSTRACT

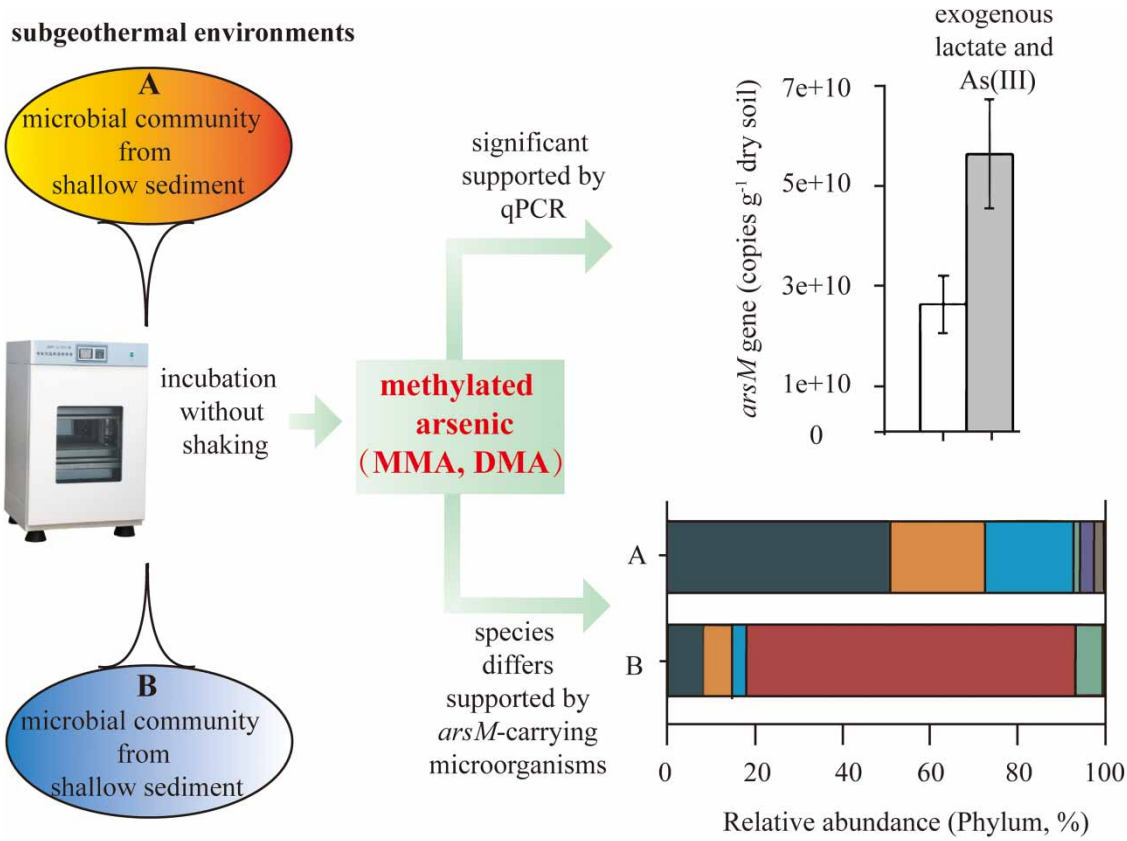
Microbially mediated arsenic methylation, remains understudied in subgeothermal environments. This study aimed to investigate the activity and diversity of *arsM*-carrying microorganisms in sediment samples (termed YC1, YC2, and YC5) from a low-temperature hot spring. Microcosm assays revealed that only YC1 and YC2 exhibited limited As-methylating activities, generating a maximum of 2.3–3.5 μg/L methylarsenate (MMA) and 2.2–2.8 μg/L dimethylarsenate (DMA). The addition of lactate and arsenite significantly promoted these activities, increasing the concentrations to 26.1–184.0 μg/L MMA and 38.1–204.0 μg/L DMA. The *arsM* gene abundance also increased by 46–276%, indicating that lactate can activate arsenite methylation. YC1 and YC2, which were closer to the hot spring hole than YC5 was, had similar patterns, and shared a similar *arsM* community structure, dominated by *Actinobacteriota, Firmicutes*, and *Proteobacteriota* on days 7 and 21. In contrast, at YC5, the sampling site far from the spring hole, representatives of *Acidobacteriota* were dominant on day 7, whereas those of *Actinobacteriota* were prevalent on day 21. *Acidobacteriota* co-occurred with dimethylarsenate production, and *Mycobacterium* co-occurred with DMA demethylation. These findings suggested that the low-temperature arsenic hot spring possessed diverse arsenic-methylating species, whereas demethylating bacteria preferred to inhabit niches farther from the hot spring.

Key words: arsenic methylation, *arsM*-carrying gene, geothermal arsenic, high-throughput sequencing, microbial community

HIGHLIGHTS

- Lactate stimulates methyl arsenic formation from subgeothermal sediments.
- The *arsM*-carrying bacterial species composition differed with distance to the hot spring.
- Demethylation occurred more often in the downstream sediment samples.

GRAPHICAL ABSTRACT



1. INTRODUCTION

Arsenic (As), a well-known human carcinogen, is ubiquitously distributed in natural environments. Prolonged interactions between geothermal fluids and reservoir host rocks, or inputs from deep magmatic components, can result in the accumulation of dissolved arsenic in geothermal systems (Wang *et al.* 2018). Arsenic concentrations in hot springs worldwide currently range from 0.01 to tens of milligrams per liter (mg/L) (Smedley & Kinniburgh 2002). The inflow of geothermal waters can result in elevated As concentrations in surface and nonthermal groundwaters. This accumulation has led to serious concerns, especially regarding the waters utilized for drinking and irrigation (Morales-Simfors & Bundschuh 2022). Microorganisms play a significant role in the biogeochemical cycling of arsenic. To date, studies involving microbiology in arsenic speciation in geothermal waters have focused primarily on arsenite [As(III)] and arsenate [As(V)]; some research has been conducted on inorganic thioarsenates and methylated thioarsenates (Hamamura *et al.* 2009; Yan *et al.* 2022; Yin *et al.* 2022; Wang *et al.* 2023). However, previous studies have shown notable concentrations of methylarsenate (MMA)/dimethylarsenate (DMA) in geothermal springs at various locations, including the Champagne Pool (New Zealand), TengChong (China), and Yellowstone National Park (USA) (Planer-Friedrich *et al.* 2007; Zhang *et al.* 2008; Hug *et al.* 2014). These organic forms substantially contribute to the overall arsenic content in geothermal waters worldwide (Wang *et al.* 2018). The microbiological and geological mechanism underlying the production of MMA/DMA in geothermal springs has yet to be fully elucidated.

The process of arsenic methylation is catalyzed by arsenite S-adenosylmethionine methyltransferase (ArsM) enzymes, the *arsM* gene of which serves as a molecular marker for exploring the diversity of arsenic-methylating microbes (Chen & Rosen 2023). Intracellularly, ArsM catalyzes the conversion of As(III) into MMA and DMA, further resulting in the generation of volatile trimethylarsine (TMAs). In this biochemical reaction, As(III) acts as a substrate, and S-adenosylmethionine acts as a methyl donor (Qin *et al.* 2009; Zhu *et al.* 2014; Roy *et al.* 2020). Converting inorganic arsenic to methylarsenic is commonly associated with a decrease in toxicity and is thus considered a detoxification mechanism (Chen & Rosen 2020). In addition,

these organic forms of arsenic (MMA and DMA) can undergo demethylation processes, leading to the conversion of inorganic arsenic compounds, specifically As(III) (Zhu *et al.* 2014). Arsenic methylation is a phenomenon observed in various organisms (Ali *et al.* 2021). Several prokaryotic strains capable of arsenic methylation, such as *Streptomyces* sp. GSRB54 (Kuramata *et al.* 2015), *Pseudomonas alcaligenes* NBRC14159 (Zhang *et al.* 2015a), *Arcticibacter tournemirensis* R1 (Zhou *et al.* 2022), *Clostridium* sp. BXM (Wang *et al.* 2015), *Paraclostridium* sp. EML (Viacava *et al.* 2022), *Bacillus* sp. CX-1 (Huang *et al.* 2018), *Arsenicibacter rosenii* SM-1 (Huang *et al.* 2016), and *Streptomyces vietnamensis* (Viacava *et al.* 2020), have been previously isolated and thoroughly studied. These strains are present in both anaerobic and aerobic environments, primarily in paddy soil and other environments, such as freshwater, mines, sludge, wetlands, and composting factories (Zeng *et al.* 2018; Qiao *et al.* 2023). Extensive research has been conducted on nongeothermal environments. However, investigations on arsenic methylation in geothermal environments are scarce. Only one report concerning Yellowstone National Park, USA, has documented the involvement of an extremophilic eukaryotic alga of the order *Cyanidiales* in this process (Qin *et al.* 2009). Recently, methanogens were proposed to drive arsenic methylation in sulfide-rich hot springs in Tengchong (Wang *et al.* 2023). However, whether the mechanism of methyl arsenic formation is similar to that of other geothermal environments remains unclear. Here, we collected sediment samples around Yanchi hot spring, which maintains an average temperature of 39 °C throughout the year. The primary aims of the present study were (i) to identify the activity and diversity of the microorganisms involved in arsenite methylation in subgeothermal environments and (ii) to evaluate the effects of distance from the hot spring on the activity and diversity of arsenite-methylating microorganisms.

2. METHODS

2.1. Site overview and sample collection

The sediment for analysis was obtained from the Yanchi hot spring (110°22′58″E, 30°26′52″N), which is the sole hot spring located in the Qingjiang River basin (Figure 1(a) and 1(b)). The Qingjiang River is a major tributary of the Yangtze River spanning 423 km. The Yanchi hot spring is located in the midsection of the Qingjiang River within the western area of Changyang Tujia Autonomous County, Yichang City, Hubei Province, China.

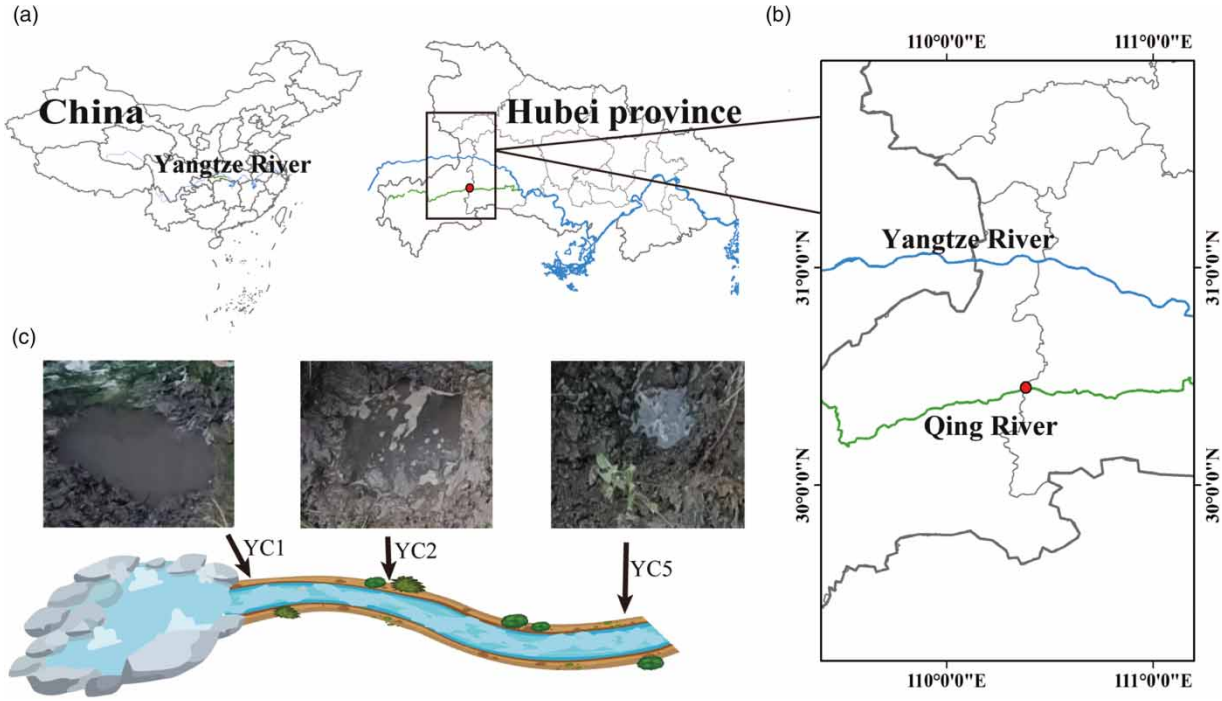


Figure 1 | Characterization of the sampling site. (a) Geographical map illustrating the location of the only hot spring in the Qingjiang River basin, Hubei Province, China. The blue curve represents the Yangtze River, and the green curve represents the Qing River. The small red circle indicates the sampling site. (b) Images depicting the sampling points located at distances of 2 m (YC1), 6 m (YC2), and 12 m (YC5) from the hot spring.

The temperature was measured using a portable thermometer (LaMotte, Chestertown, MD, USA). The central part of the hot spring maintained a steady temperature of 42 °C, whereas the temperature of the water discharged from the spring was 39 °C. The aforementioned temperatures are year-round. Nine samples were obtained from three distances, and three replicate samples were collected within a 0.2-m range within each of these distances. The sampling locations were 2 m (referred to as YC1), 6 m (referred to as YC2), and 12 m (referred to as YC5) from the hot spring (Figure 1(c)). All the samples were collected at a depth of 15–25 cm below the ground surface. These samples were then promptly placed into sterilized tubes and kept chilled with ice during transport. All the samples reached the laboratory for further analysis within a 12-h timeframe.

2.2. Chemical analysis

Concentrations of arsenic, including total arsenic, soluble arsenic, MMA, and DMA, were assessed utilizing high-performance liquid chromatography coupled with atomic fluorescence spectrometry (HPLCAFS9600). Specifically, an Agilent 1220 HPLC system equipped with a Hamilton PRP-X100 ion exclusion column was utilized following the methods described in previous studies (Zeng *et al.* 2018). The mobile phase consisted of 15.0 mM $(NH_4)_2HPO_4$, and the flow rate was set at 1.0 mL/min. The retention times for As(III), DMA, MMA, and As(V) were determined to be 2.58, 3.59, 4.62, and 9.62 min, respectively (Georgiadis *et al.* 2006). An ion chromatography system (DX-120, Dionex, USA) was used to measure the anion concentrations (Broderick *et al.* 2005). For the quantification of metal ion concentrations, ICP-AES (inductively coupled plasma-atomic emission spectrometry) was performed using an IRIS Intrepid II XSP instrument manufactured by Thermo Fisher, USA (Chen *et al.* 2017). The total organic carbon (TOC) and total nitrogen (TN) contents were determined using a carbon/nitrogen analyzer (PRIMACS™ SNC-100, The Netherlands).

2.3. Microcosm incubation

Microcosm incubation analyses were also conducted to assess the arsenic methylation activity of the microbial community in the sediment samples. These experiments were performed in triplicate to ensure reliability. In the experimental group, each active microcosm consisted of 50.0 g of sediment and 50.0 mL of mineral salt medium (Bahar *et al.* 2012). The experimental group was divided into two subgroups. One subgroup was left untreated for the detection of microbial methylation activity under natural conditions. The other subgroup was treated with 0.1 mM As(III) and 25.0 mM lactate in a 100-mL container. As(III) served as the substrate, and lactate acted as the carbon source for the methylation reaction. The control mixtures were subjected to autoclaving. All microcosms were incubated at 39 °C without shaking. Approximately 1.5 mL of the cultures were collected from each vial on days 7 and 21 of the incubation period and subjected to quantitative analysis of MMA and DMA concentrations using HPLCAFS9600.

2.4. Quantitative PCR assay

Total DNA extraction was performed from soil samples (0.5 g) that had undergone 7- and 21-day incubations using the Soil DNA Extraction CZ Kit (DC306, FINDROP, China). The DNA concentrations were detected with a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The quantification of the *arsM* gene abundance was achieved with the CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) (Zhao *et al.* 2013). The 20-μL qPCR mixture consisted of 10 μL of SYBR™ Green Master Mix (Thermo Fisher Scientific, USA), 0.4 μL of passive reference dye, 0.4 μL of each primer (10 mM), 1.5 μL of template DNA, and 7.3 μL of sterile water without DNA. The qPCR program included an initial denaturation step at 94 °C for 5 min, followed by 40 cycles of amplification (94 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s). A linear standard curve with $R^2 > 0.99$ was obtained in the quantitative assay. The amplification efficiency was within the range of 90–110%. A single peak was observed in the melt curve analysis. Each sample was analyzed in triplicate to ensure reproducibility. The primers used for *arsM* gene amplification are presented in Table 1.

2.5. High-throughput sequencing

The V3–V4 hypervariable regions of the *arsM* genes were amplified with the arsMF and arsMR primers (Table 1) (Jia *et al.* 2013). The amplicons were purified and quantified. The V3–V4 regions were subsequently sequenced using the Illumina NovaSeq PE250 platform (Magigene Biotechnology Co., Ltd, China). The raw data obtained were subjected to processing, which included demultiplexing based on barcode sequences and subsequent quality filtering (Wang *et al.* 2023). Quantitative Insights Into Microbial Ecology (QIIME, V1.9.0) software was utilized to filter and assemble the raw sequences. Operational

Table 1 | PCR primers

arsM genes	Primer sequence (5′-3′)	References
Forward	TCYCTCGGCTGCGGCAAYCCVAC	Jia *et al.* (2013), Zhang *et al.* (2015b)
	GTGCTCGAYCTSGGCWCCGGC	
	GGCATCGACGTGCTKCTBTCSGC	
Reverse	AGGTTGATGACRCAGTTWGAGAT	
	CGWCCGCCWGGCTTWAGYACCCG	
	GCGCCGGCRAWGCAGCCWACCCA	

taxonomic units (OTUs) were defined at a 97% similarity threshold using UPARSE (version 7.1) and checked for chimeric sequences with UCHIME (Wang *et al.* 2023).

2.6. Phylogenetic analysis

Using the BLAST server, a search of the GenBank database was launched to identify homologous protein sequences (http://blast.ncbi.nlm.nih.gov/). We further conducted multiple sequence alignments with ClustalW2 software (https://www.ebi.ac.uk/Tools/msa/clustalw2/). A phylogenetic tree was subsequently constructed through neighbor-joining analysis with MEGA 11.0.13 (https://megasoftware.net/dload_win_beta) following the methodology outlined in a prior study (Zhou *et al.* 2022). The reliability of the phylogenetic tree was determined by performing 1000 bootstrap replications.

3. RESULTS

3.1. Characterization of the sampling site

Table 2 presents a summary of the geochemical properties of the sediment samples. The total arsenic concentration in the sediments ranged from 0.090 to 0.128 mg/kg, which is significantly lower than the concentrations previously found in sediments from other hot springs, such as Tengchong (1.6–6406 mg/kg) (Yan *et al.* 2022), the Tibetan Plateau (101.9–263.7 mg/kg) (Zhang *et al.* 2017), and Latin America (0.7–111 mg/kg) (Morales-Simfors *et al.* 2020). Additionally, we

Table 2 | Characteristics of the sediment samples from the Yanchi hot spring

	Sediment samples		
Parameters	YC1	YC2	YC5
pH	8.33	8.35	8.49
Total As (mg/kg)	0.112	0.128	0.090
Soluble As (μg/kg)	0.042	0.057	0.028
TOC (g/kg)	5.56	18.70	6.46
TN (g/kg)	0.88	1.53	0.52
NO_3^- (mg/kg)	26.80	45.80	7.55
NH_4^+ (mg/kg)	3.53	3.66	2.44
SO_4^{2-} (mg/kg)	136.00	151.00	110.00
Cl^- (g/kg)	0.666	0.732	0.355
K (g/kg)	17.3	21.7	12.5
Ca (g/kg)	96.4	94.6	173.0
Na (g/kg)	2.52	3.00	2.21
Mg (g/kg)	8.82	8.95	8.79
Fe (g/kg)	21.6	25.2	17.0
Mn (g/kg)	335	349	259

established that the sediments contained TOC ranging from 5.56 to 18.70 g/kg and TN ranging from 0.88 to 1.53 g/kg. These components play pivotal roles as essential energy sources, providing both carbon and nitrogen for microbial growth. The sediment also contained sulfate (110.00–136.00 mg/kg), ammonium (2.44–3.66 mg/kg), and nitrate (7.55–45.80 mg/kg).

3.2. Microbial arsenic methylation activity in shallow sediments

As shown in Figure 2, the autoclaved sediment slurries had negligible DMA/MMA levels, whereas the nonsterilized cultures from the sediment samples of YC1 and YC2 had slightly greater arsenic methylation levels. However, no soluble methylated arsenic was detected in the nonsterilized sample YC3. Specifically, the MMA content in sample YC1 remained relatively stable at 3.5 μg/L on day 7 and at 3.4 μg/L on day 21. On the other hand, the DMA level decreased from 2.8 μg/L on day 7 to undetectable levels on day 21. For sample YC2, MMA was detected only on day 7 at a concentration of 2.3 μg/L, whereas DMA was detected solely on day 21 at a concentration of 2.2 μg/L. Overall, the arsenic methylation activity in the three samples was minimal or even undetectable, indicating a weak capacity of the microbial community in the hot spring sediments to methylate arsenic under natural conditions at 39 °C.

Given the limited TOC content in the sediment, we investigated microbial arsenic methylation activity by introducing additional organic carbon. As depicted in Figure 2, methylarsenic was detected in samples YC1 and YC2 on both days 7 and 21, at which time the MMA levels were significantly greater than the DMA levels. In YC1, the MMA concentrations were 63.5 and 169.0 μg/L on days 7 and 21, respectively, whereas the corresponding DMA concentrations were only 36.5 and 38.1 μg/L, respectively. Similarly, in YC2, the MMA content was 184.0 and 106.0 μg/L on days 7 and 21, respectively, with DMA concentrations of only 36.0 and 41.9 μg/L on the corresponding days. However, in contrast to YC1 and YC2, YC5 had higher DMA levels than did MMA, which were detected only on day 7, with concentrations of 26.1 μg/L for MMA and 204.0 μg/L for DMA. Overall, all three samples showed significantly stronger methylarsenic activity when

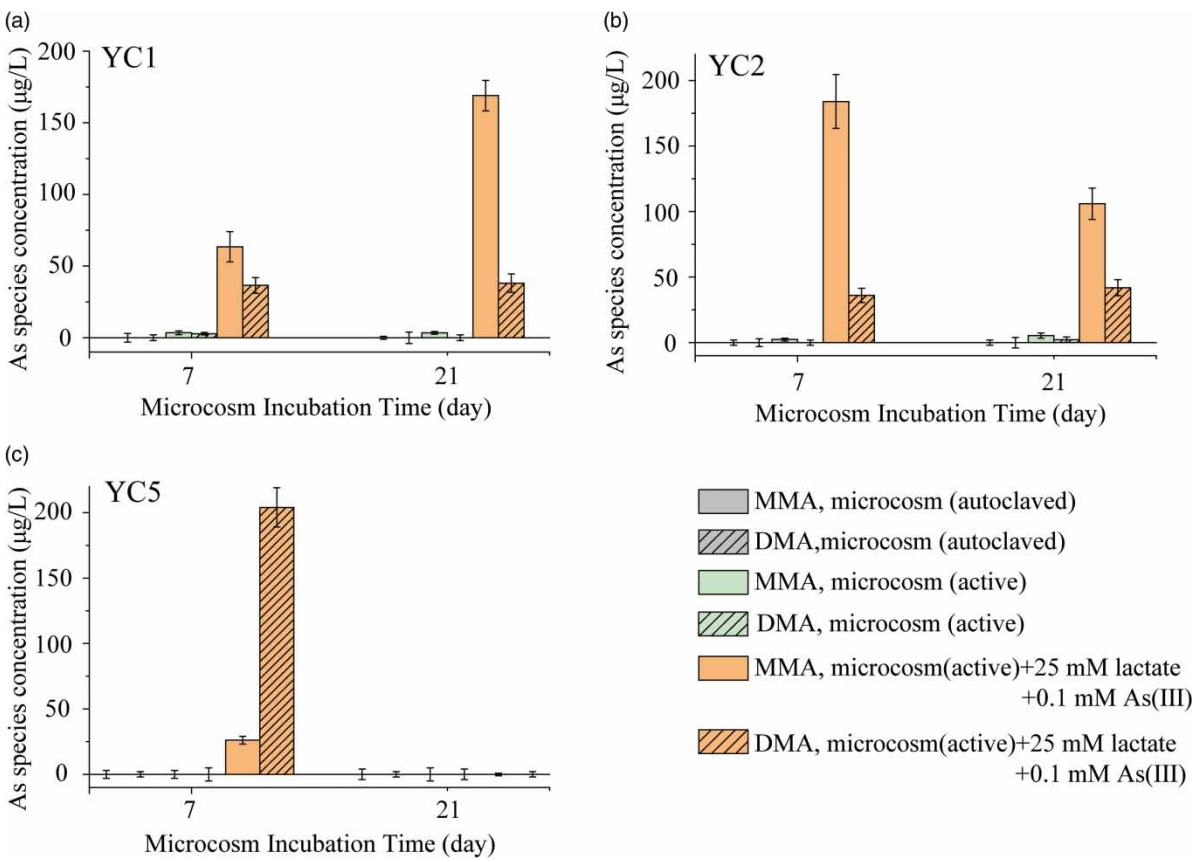


Figure 2 | Arsenic methylation activities of microbial communities from three sediment samples with or without exogenous lactate and As(III). The concentrations of methylated arsenic in the microcosms of YC1 (a), YC2 (b), and YC5 (c) during a 21-day incubation period.

supplemented with exogenous carbon and arsenic than under natural conditions, indicating the activation of arsenic methylation microorganisms. Notably, the methylarsenic content in YC1 increased over time, whereas that in YC2 and YC5 decreased.

3.3. Abundance of *arsM* genes

Since microbial processes drive primarily arsenic methylation in the soil, we evaluated the abundance of key genes responsible for these processes during 7 days of incubation. Lactate and arsenite addition considerably increased *arsM* gene abundance in the microcosms (Figure 3). In samples YC1, YC2, and YC5, the *arsM* gene abundance was significantly greater than that observed under natural conditions, showing increases of 116, 276, and 46%, respectively. Therefore, the presence of lactate and arsenite strongly stimulated the growth of *arsM*-containing bacteria in the soil, as determined from the sediment samples, enhancing the concentration of MMA/DMA in the soil.

3.4. Diversity of *arsM*-carrying microorganisms in sediments

To gain a comprehensive understanding of the microbial basis contributing to our results, we examined the community of *arsM*-carrying prokaryotic microorganisms found in both the natural sediment and the treatment sediments collected on days 7 and 21. We identified a total of 2,091 OTUs corresponding to *arsM*-carrying bacteria. These OTUs were classified into 22 phyla, 183 families, and 586 species. Additionally, we discovered 30 OTUs of *arsM*-carrying archaea, which were assigned to two phyla, five families, and nine species (Table 3). A rarefaction analysis conducted using QIIME demonstrated that these sequences covered more than 99.84% of the *arsM*-carrying microorganisms in the sediments (Table 3; Figure S1).

The community structures of the *arsM*-carrying microorganisms in the sediment varied among the different treatment samples at both the phylum and genus levels. However, the *arsM* gene communities in YC1 and YC2 were similar at the phylum level (Figure 4(a)). The dominant phyla in both YC1 and YC2 on days 7 and 21 were *Actinobacteriota* (15.8–60.2%), *Firmicutes* (14.3–51.3%), and *Proteobacteriota* (13.0–21.0%), which accounted for more than 70% of the combined relative abundances. Other phyla with relative abundances exceeding 0.01% included *Chloroflexi*, *Acidobacteriota*, *Planctomycetes*, *Euryarchaeota*, *Verrucomicrobia*, *Bacteroidetes*, *Nitrospirae*, and *Spirochaetes*. Previous studies have highlighted the importance of the *Proteobacteriota*, *Firmicutes*, and *Actinobacteriota* in arsenic methylation (Zhang *et al.* 2015b; Zhai *et al.* 2017; Qiao *et al.* 2023), as they are commonly associated with *arsM* gene-carrying microbial communities (Figure S2).

At the genus level, there were slight differences in the community of *arsM*-carrying microorganisms between YC1 and YC2 (Figure 4(b)). In YC1, the genera with the highest abundance on day 7 were *Bacillus*, *Bilophila*, and *Geobacillus*, whereas on day 21, the genera with the highest abundance were *Parageobacillus*, *Desulfosporosinus*, *Sorangium*, and *Rhizobium*. In YC2, the genera with the highest abundance on day 7 were *Geobacillus*, *Caenispirillum*, *Caldilinea*, *Sulfuricella*, *Methanosarcina*, *Thioalkalivibrio*, and *Pseudomonas*, whereas on day 21, the genera with the highest abundance were *Rhodopseudomonas*, *Alkaliphilus*, *Ktedonobacter*, *Thiobacillus*, *Cellulomonas*, *Mycobacterium*, and *Pseudomonas*.

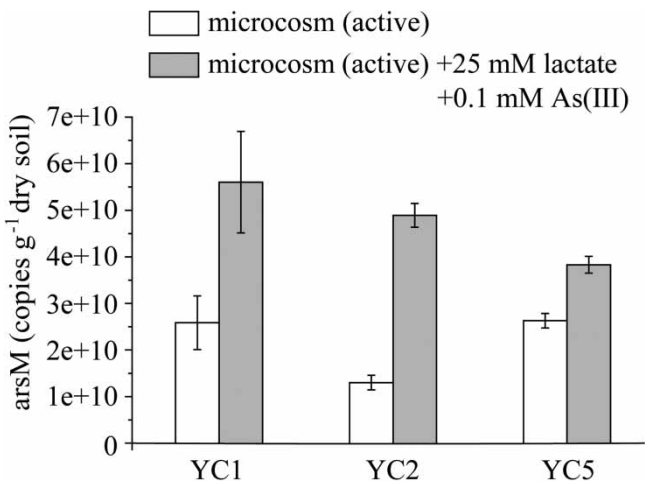


Figure 3 | Changes in the abundance of the *arsM* gene with and without exogenous lactate and As (III) after 7-day incubation.

Table 3 | Diversity and richness of the *arsM*-carrying microbial communities

Sample	Index				
	Chao1	ACE	Simpson	Shannon	Coverage (%)
YC1-C	358.4	411.6847	0.146	3.8	99.94
YC1-7	543.3	604.6924	0.133	4.1	99.91
YC1-21	491.6	576.2286	0.133	4.05	99.89
YC2-C	494.4	580.6317	0.118	4.03	99.89
YC2-7	593.3	672.8753	0.134	4.18	99.88
YC2-21	561.3	626.7159	0.192	3.6	99.90
YC5-C	688.1	737.9108	0.0704	4.74	99.91
YC5-7	316.4	372.2407	0.568	1.9	99.94
YC5-21	654.1	690.8322	0.248	3.63	99.84

C refers to samples without treatment; 7 refers to day 7; 21 refers to day 21.

In contrast, the *arsM* gene community in YC5 was completely different from that in YC1 and YC2. *Acidobacteriota* dominated at the phylum level in YC5 on day 7, accounting for 75.0% of the community, followed by *Actinobacteriota* (7.3%) and *Firmicutes* (6.6%). Subsequently, on day 21, the abundances of *Actinobacteriota* and *Proteobacteriota* increased significantly, composing 60.5 and 14.0%, respectively, of the community (Figure 4(a)). YC5 exhibited a pattern dissimilar from that of YC1 and YC2 at the genus level (Figure 4(b)). On day 7, reads from the unassigned genus emerged, and further sequence analysis identified these reads as members of the *Acidobacteriaceae* family within the *Acidobacteriota* phylum. Notably, on day 21, the proportion of *Mycobacterium* dramatically increased to 60.1%, a tenfold increase compared to its 5.9% abundance on day 7 (Figure 4(b)). Several other genera with relatively smaller proportions included *Geobacillus* (1.7%), *Rhodopseudomonas* (2.4%), *Desulfotomaculum* (3.1%), *Desulfococcus* (6.0%), and *Blastopirellula* (3.1%).

Additionally, we discovered five best-matched *arsM*-carrying methanogens, namely, *Methanosphaerula palustris*, *Methanosarcina barkeri*, *Methanoculleus marisnigri*, *Methanosarcina mazei*, and *Methanosarcina acetivorans*, in the three samples (Figure S3).

4. DISCUSSION

Arsenic biogeochemical processes include oxidation, reduction, arsenic sulfide reactions, and methylation. However, to date, research on geothermal environments has been predominantly concentrated on the former three processes. Studies on arsenic methylation have largely centered on its role as a detoxification mechanism in prokaryotic and eukaryotic microbes (Chen *et al.* 2019; Viacava *et al.* 2020). Nevertheless, limited research has been performed on *arsM* gene activity and diversity. Furthermore, these studies have been predominantly conducted in nongeothermal environments, such as rice paddies. With an average temperature of 39 °C throughout the year, the Yanchi hot spring provides ideal conditions for studying arsenic-methylating microorganisms in a geothermal environment. In the present study, we found that the sediment along the outflow channel of the hot spring exhibited arsenic methylation capacity. Additionally, arsenic-demethylating species were detected, primarily favoring a niche farther away from the hot spring hole. These observations contribute to advancing our understanding of microorganism-mediated arsenic methylation in geothermal settings.

YC1 and YC2, the two sampling sites closest to the hot spring hole, had significantly greater MMA levels than did DMA on both days 7 and 21 when lactate and arsenite were supplemented. These results were likely due to the similar compositions of the *arsM* microbial communities (Zhao *et al.* 2013; Zhai *et al.* 2017). *Actinobacteriota*, *Firmicutes,* and *Proteobacteriota* were dominant in the bacterial community harboring *arsM* in YC1 and YC2. Moreover, *Methanosarcina* exhibited a relatively greater abundance on day 7 in the YC1 and YC2 samples. *Methanosarcina* sp. can methylate As(III), predominantly generating MMA and DMA to a lesser extent, which cannot be demethylated. Specifically, the arsenic methylation abilities of *Methanosarcina mazei* Gö and *Methanosarcina acetivorans* C2A have been extensively studied (Thomas *et al.* 2011; Wang *et al.* 2014), and their relatives were also detected in this study (Figure 4, Figure S3).

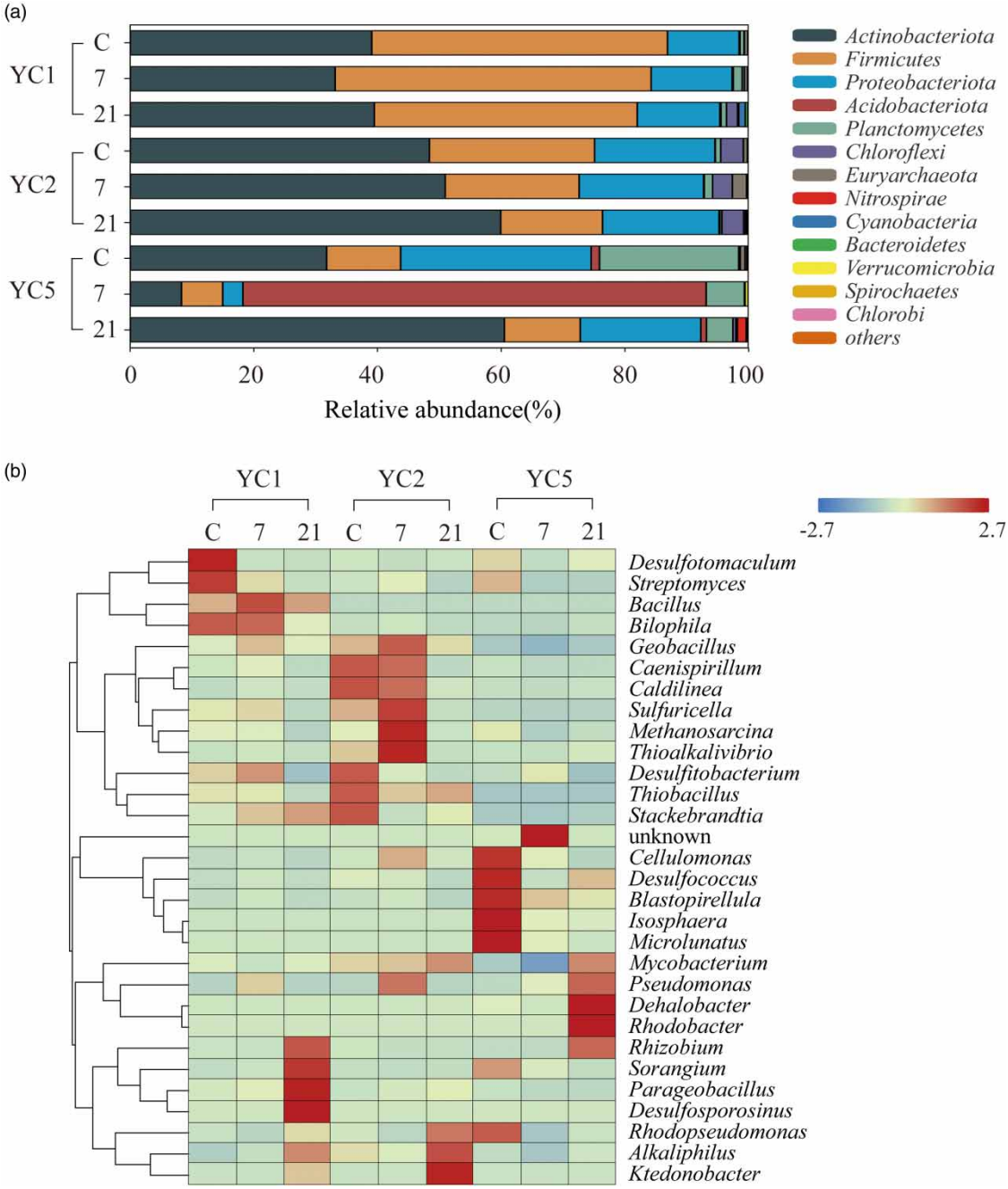


Figure 4 | Dynamic changes in the *arsM*-carrying community during incubation in the three samples. (a) Relative abundances of *arsM*-harboring As (III) methylators at the phylum level, with a relative abundance threshold of 0.01% or higher. (b) Heatmap representing the top 30 abundant genera. The horizontal axis represents the samples, the vertical axis represents the species, and the clustering tree on the left illustrates the species clustering. The middle square corresponds to the normalized *Z*-value of the species' relative abundance in each row, with red colors indicating higher abundance within the samples. C refers to samples without treatment; 7 refers to day 7; 21 refers to day 21.

The difference in methylarsenic production between the YC1 and YC2 microcosms on day 21 compared to day 7 can be attributed to specific microbial enrichment. In YC1, the relative enrichment of *Bacillus* on day 21 led to greater levels of methylarsenic bacteria than those on day 7. A previous study showed that the purified S-adenosylmethionine methyltransferase of *Bacillus* sp. CX-1 efficiently methylated As(III), with DMA produced as the final product (Figure S2) (Huang *et al.* 2018), indicating the accumulation of methylarsenic rather than its volatilization. In contrast, the decrease in methylated

arsenic observed in YC2 on day 21 compared to day 7 may be due to the greater relative enrichment of *Rhodopseudomonas*. *Rhodopseudomonas* species have been reported to promote arsenic methylation and volatilization, as they have been identified as active arsenite-methylating organisms in paddy soils (Figure S2) (Qin *et al.* 2006; Chen *et al.* 2014; Zhao *et al.* 2015). Due to continuous arsenic volatilization, it was reasonable to see that the MMA and DMA levels decreased on day 21 in YC2. Interestingly, some *arsM*-carrying bacteria in YC2 were also found to be sulfate reducers or oxidizers, such as *Sulfuricella*, *Desulfitobacterium*, and *Thioalkalivibrio* (Sorokin *et al.* 2012; Li *et al.* 2018; Kojima *et al.* 2021). This result suggested that the cycling of arsenic in this hot spring may also couple with S cycling. This finding is further supported by the highest recorded sulfate level of 152 mg/kg in the geochemical parameters of YC2 (Table 2).

On day 7, the DMA level in the microcosm from YC5 was initially greater than that in the microcosm from MMA. However, both the DMA and MMA levels decreased to undetectable levels by day 21. The community of microorganisms harboring the *arsM* gene in YC5 was distinct from that in YC1 and YC2, with a notable abundance (75.0%) of *Acidobacteriota* observed on day 7. *Acidobacteriota* have been widely associated with arsenic methylation processes (Figure S2) (Yan *et al.* 2022). Subsequently, on day 21, the dominance of *Actinobacteriota* (60.5%) and *Proteobacteriota* (14.0%) increased. The *Proteobacteriota*, which carry the *arsM* gene, are key players in arsenic methylation and volatilization (Figure S2) (Bennett *et al.* 2012), as shown at the genus level by the observed increases in the abundances of *Pseudomonas*, *Dehalobacter*, and *Rhodobacter* (Figure 4(b)). Previously, *Pseudomonas alcaligenes* NBRC14159 was found to rapidly methylate As(III) to DMA and trimethylarsine oxide (TMAO) (Figure S2) (Zhang *et al.* 2015a). More importantly, an approximately tenfold increase was observed in the proportion of *Mycobacterium*, affiliated with *Actinobacteriota*, from 5.9 to 60.1%. *Mycobacterium* species are known for their strong ability to demethylate methylarsenic species (Lehr *et al.* 2003; Zhu *et al.* 2017). Consequently, on day 21, almost no detectable levels of methylarsenic were found in our study.

Furthermore, the phylum-level abundance of *arsM*-carrying bacteria within these three microbial community samples differed from what was observed in the Tengchong Hot Springs (Wang *et al.* 2023). Additionally, the five identified methanogen species in our samples differed from the nine best-matched *arsM*-carrying methanogens previously reported in the Tengchong hot spring (Wang *et al.* 2023); these included *Methanosarcina horonobensis*, *Methanosarcina thermophila*, *Methanolacinia petrolearia*, *Methanolacinia paynteri*, *Methanoculleus taiwanensis*, *Methanoculleus thermophilus*, *Methanocella paludicola*, *Methanolobus halotolerans*, and *Methanothrix soehngenii*.

Our study exclusively investigated microbial communities harboring the *arsM* gene in geothermal environment samples stimulated by lactate. However, it remains unclear exactly how many types of microorganisms, and under what conditions, participate in the actual arsenic methylation process *in situ*. Metagenomic and transcriptomic data should be integrated into future research on the composition and ecophysiology of active microbial functional groups involved in arsenic methylation and demethylation under various geothermal conditions. This approach could aid in elucidating the mechanisms of microbial-driven arsenic biogeochemical processes in actual geothermal environments. Additionally, understanding the microbial mediation of arsenic biogeochemical cycles in geothermal ecosystems could enhance our ability to manage or manipulate specific microbial functional groups in certain environments. Biological indicators or mechanisms identified through this research could be instrumental in signaling or remediating arsenic-contaminated environments.

5. CONCLUSIONS

In this study, we discovered arsenic-methylating species in the sediments of a low-temperature thermal spring. Arsenic demethylating species were also detected in a niche located farther from the hot spring hole. The microbes in the two sediments near the hot spring hole continuously methylated arsenite after stimulation with lactate and arsenite supplementation, sharing similar *arsM*-carrying communities. Sediments slightly farther from the hot spring showed both methylating and demethylating activities. The latter were enriched in As-demethylating species such as *Mycobacterium*. These findings enhance the existing understanding of microorganism-mediated arsenic methylation in low-temperature, low-arsenic geothermal environments.

AUTHOR CONTRIBUTIONS

Y.Y. and C.X.M. conceived and designed the experiments and conducted the experiments. Y.Y. and Y.L.Y. analyzed the data. The manuscript was written by Y.Y. Y.Y. and Y.L.Y. contributed to the provision, use, and description of the reagents/materials/analysis tools.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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