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Efficient Arsenic Methylation and Volatilization Mediated by a Novel Bacterium from an Arsenic-Contaminated Paddy Soil

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Abstract

Microbial arsenic (As) methylation and volatilization are important processes controlling the As biogeochemical cycle in paddy soils. To further understand these processes, we isolated a novel bacterial strain, SM-1, from an As-contaminated paddy soil. SM-1 showed strong As methylation and volatilization abilities, converting almost all arsenite (10 μ M) to dimethylarsenate and trimethylarsenic oxide in the medium and trimethylarsine gas into the headspace within 24 h, with trimethylarsine accounting for nearly half of the total As. On the basis of the 16S rRNA sequence, strain SM-1 represents a new species in a new genus within the family *Cytophagaceae*. Strain SM-1 is abundant in the paddy soil and inoculation of SM-1 greatly enhanced As methylation and volatilization in the soil. An arsenite methyltransferase gene (*ArarsM*) was cloned from SM-1. When expressed in *Escherichia coli*, *ArarsM* conferred the As methylation and volatilization abilities to *E. coli* and increased its resistance to arsenite. The high As methylation and volatilization abilities of SM-1 are likely attributed to an efficient *ArarsM* enzyme coupled with low arsenite efflux. These results suggest that strain SM-1 plays an important role in As methylation and volatilization in the paddy soil and has a great potential for As bioremediation.

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b01974.

Tables showing the composition of culture media and primers for PCR. Figures showing As species changes in soil porewater during incubation, electron micrographs of strain SM-1, a phylogenetic tree of 16S rRNA sequences of strain SM-1 with representative members of the family *Cytophagaceae*, multiple alignment and phylogenetic analysis for arsenite methyltransferases (*ArsMs*) from seven species, the expression of *ArarsM* in strain SM-1 in response to As(III) or As(V) exposure; HPLC–ICP–MS chromatograms of soluble and volatile As species in the *E. coli* experiments; and the heterologous expression of *ArarsM* enhances As(III) resistance in *E. coli* strain AW3110. The Supporting Information is available free of charge on the ACS publications Web site. (PDF)

The authors declare no competing financial interest.

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Graphical Abstract

INTRODUCTION

Arsenic (As) is a toxic metalloid and a nonthreshold Group 1 carcinogen to humans.¹ Arsenic is ubiquitous in the environment and derived from both geogenic and anthropogenic sources. Millions of people worldwide suffer from chronic As poisoning, especially in south and southeast Asia.² Humans are exposed to As mainly through drinking water and food. Rice, the staple food for more than half of the world's population, is a major source of dietary As for populations in south and southeast Asia.^{3–5} Large areas of paddy soils in south and southeast Asia are contaminated with As due to mining, smelting, irrigation with high-As groundwater, and the use of As-containing agrochemicals.^{2,6,7} It is therefore important to understand the biogeochemical cycling of As in paddy systems.

Episodic flooding and draining of paddy soil during rice cultivation have profound impact on the As biogeochemical cycling. Upon flooding, soil redox potential decreases, leading to reductive dissolution of iron oxides and hydroxides together with the adsorbed arsenate [As(V)], which is then reduced by microorganisms to arsenite [As(III)].^{8,9} In addition, adsorbed As(V) can be reduced to As(III); the latter is less strongly adsorbed and has a greater tendency to partition into the soil solution phase.^{8–10} Flooding of paddy soil thus results in increased bioavailability of As to rice plants.^{11,12} Another important change upon flooding of paddy soil is that microbial As methylation is enhanced.^{13,14} This could be because As(III), the substrate of As methylation, is mobilized or anaerobic microorganisms capable of As methylation become more abundant.^{13,15} Microbial As methylation is an important component of the global biogeochemical cycle of As¹⁶ and is also a prerequisite for the production of volatile methylarsine gases.^{17,18} The biovolatilization of As from the terrestrial environment is estimated to range from hundreds to tens of thousands ton per annum, but the pathway is poorly understood.¹⁸ Microbial As methylation in paddy soil also impacts As speciation in rice grain. Rice grain contains both inorganic and organic (methylated) As species, with methylated As species accounting for between 10% and 90% of the total As in rice grain depending on the geographical region and the growth conditions of rice.^{15,19,20} Methylated As species in rice are derived from soil microorganisms because rice plants do not appear to be able to methylate As.²¹

Many microorganisms are able to methylate As, and some are also able to volatilize As.²² Arsenic methylation is catalyzed by As(III) *S*-adenosylmethionine (SAM) methyltransferase enzymes (ArsM), which transfer methyl group from SAM to As(III) to produce mono-, di-, and trimethyl arsenical compounds.^{17,23} Depending on the microorganism studied, a range of different volatile or nonvolatile methylated As compounds are produced.^{17,23–28} Genes encoding ArsM appear to be abundant and diverse in paddy soils,^{29,30} but to date, only a few studies have investigated microbial isolates from paddy soils for their As methylation abilities. Kuramata et al.²⁷ isolated an aerobic bacterium belonging to *Streptomyces* sp. from a paddy rhizosphere soil and showed that it can methylate As(III) to methylarsenate [MAs(V)] and dimethylarsenate [DMAs(V)]. Wang et al.²⁶ reported that an anaerobic sulfate-reducing bacterium belonging to *Clostridium* sp. isolated from a paddy soil also methylates As(III) to MAs(V) and DMAs(V). Both isolates appeared to produce very little volatile As species. In both laboratory and field studies, methylarsine gases, especially TMAs(III), have been detected from paddy soils,^{13,14} but the microorganisms mediating As biovolatilization remain unknown.

In the present study, we isolated a novel bacterial strain SM-1 from an As-contaminated paddy soil. The strain represents a new genus in the family of *Cytophagaceae* and has a strong ability to methylate and volatilize As. Here, we characterize the molecular mechanisms underpinning the high As methylation and volatilization in strain SM-1 and its role in As biogeochemical cycle in paddy soil.

MATERIALS AND METHODS

Soil Incubation and As Speciation in Porewater

A paddy soil was collected from Shimen City in the Hunan Province in southern China. The soil is moderately contaminated with As (30.0 mg kg⁻¹) due to mining activities nearby. The soil contains 11.1 g kg⁻¹ of organic carbon and has a pH of 6.85. The soil was air-dried, sieved to <2 mm, and stored in the dark. A total of 500 g of soil was placed into each black plastic bottle and flooded with double-distilled water to form a 3 cm layer of standing water. A total of two treatments were included: a normal static culture with air in the headspace and an anaerobic culture using N₂ to flush the bottles and fill the headspace. Each treatment had four replicates. A Rhizon soil solution sampler was used on days 1, 7, 14, 21, 30, 45, and 60 after flooding to collect the soil porewater, which was acidified to pH < 2.0 with 1% (v/v) HCl to preserve As species.²⁹ As speciation analysis was performed using high-performance liquid chromatography–inductively coupled plasma mass spectrometry (HPLC–ICP–MS) as described previously.³¹ Samples were analyzed directly for the quantification of As(V), MAs(V), and DMAs(V) and after reaction with 20% (v/v) H₂O₂ to oxidize As(III) to As(V) to separate and quantify the coeluted TMAs(V)O and As(III).

Isolation of As-Methylating Bacteria

After the soil was flooded for 30 d, 2 g of soil was collected from the surface layer (about 0.1 cm below the standing water) and transferred to a conical flask containing 100 mL of ST10⁻¹ medium (composition given in Table S1). The flask was incubated in a shaker (200 rpm) at 28 °C for 1 week. The enrichment culture was centrifuged at 12000 rpm. The

supernatant was filtered through 0.22 μm filter membrane and analyzed for As species by HPLC–ICP–MS. The enrichment culture was diluted and spread onto ST10⁻¹ agar medium plates. After incubation for 7 d, colonies with different morphological characteristics were selected and inoculated in 3 mL of ST10⁻¹ liquid medium with 10 μM As(III) in glass test tubes. After another 4 d of growth, As species in the medium were determined. A single strain (SM-1) with As(III) methylation ability was isolated, purified, and stocked with 30% glycerol at -80°C .

Arsenic Methylation by Strain SM-1

The As methylation ability of strain SM-1 was assayed in a time-course experiment. A single colony of this isolate was inoculated into 5 mL of R₂A medium (Table S1) to an OD₆₀₀ of 1.0. The cells were diluted 100-fold into 100 mL of fresh R₂A medium containing 10 μM As(III). The cultures were incubated for 24 h at 37 $^\circ\text{C}$ in a shaker. Aliquots of the culture medium were taken every 4 h for analysis of As species. At the same time points, volatile As compounds were purged from the headspace for 15 min and trapped using an AgNO₃ impregnated silica gel tube, and As species were analyzed as described before.³¹ A similar experiment was conducted with 10 μM As(V) in R₂A medium (containing 2.1 mM total P) or in R₂A medium minus the addition of phosphate (containing 0.57 mM total P). Volatile As was purged every 4 h as in the As(III) experiment.

Strain Identification

The total DNA of the isolate was purified by using CTAB extraction.³² The 16S rRNA gene was amplified from genomic DNA by PCR using rTaq DNA polymerase (Takara) with the primer pair 27F/1492R (Table S2). The PCR amplicons were purified using a DNA Gel Extraction Kit (Axygen) and cloned into PMD18-T vector (Takara) for gene sequencing in Genscript. The 16S rRNA gene sequence (1470 bp) obtained was deposited into the GenBank database under the accession no. KT989310. The sequence of strain SM-1 was compared and aligned with other 16S rRNA gene sequences in the EzTaxon database (<http://www.ezbiocloud.net/eztaxon>) and used to build a neighborjoining phylogenetic tree using soft MEGA (version 5.0).

Quantification of Strain SM-1 16S rRNA Copy Number

To design a specific primer pair for quantitative real-time PCR, we first aligned the 16S rRNA gene of strain SM-1 with other 16S rRNA gene sequences downloaded from EzTaxon database (<http://www.ezbiocloud.net/eztaxon>) by ClustalX program. The strain SM-1 specific sequence regions were detected and used for designing primer pairs using soft Primer Premier 5. The specificity of designed primer pairs were verified using the ProbeCheck program (<http://131.130.66.200/cgi-bin/probecheck/content.pl?id=home>). The primer pair P1–P2 (Table S2) was confirmed to be highly specific because it is not complementary to any bacterial sequences other than strain SM-1. The copy number of SM-1 16S rRNA was determined by quantitative real-time PCR analysis with this genus-specific primer pair as described previously.³³

Effect of SM-1 Inoculation on As Methylation and Volatilization in Soil Slurry

To investigate the role of strain SM-1 in As methylation in the Shimen paddy soil, we conducted a soil-slurry experiment as described by Chen et al.³⁴ with some modification. Briefly, 100 g of unsterilized soil were added to a 250 mL flask and flooded with 100 mL of double-distilled water. The strain SM-1 was cultured overnight at 37 °C in R₂A medium to the exponential phase. The bacterial culture (20 mL) was added to the flask. Treatments included with or without SM-1 inoculation in combination with or without the addition of 20 µM As(III). A total of two controls were included, one with sterilized soil and the other with unsterilized soil but without strain inoculation; both were treated with an equal volume of R₂A medium. There were four replicates for each treatment. All flasks were incubated for 5 d in a shaker at 37 °C. Soil slurry (10 mL) was sampled from each flask and centrifuged at 12000 rpm. The supernatants were used for As speciation analysis, and the soil was collected for subsequent quantification of the 16S rRNA copy number of strain SM-1. Volatile As gases from each flask were trapped and quantified as described above.

Cloning and Functional Characterization of *arsM* in Strain SM-1

The genome of strain SM-1 was sequenced using Illumina PE sequencing technology. A putative *arsM* gene (*ArarsM*) was identified in the draft genome of strain SM-1 by functional gene annotation. The complete nucleotide sequence of this gene has been deposited into the GenBank database (no. KU641426) and was compared with other sequences in the NCBI database using the Blast option. Multisequence alignments and phylogenetic analysis with other typical ArsMs were performed using the software DNAMAN.

To characterize the function of *ArarsM*, we constructed expression vectors pET29a-*ArarsM* and pET29a-*RparsM* and expressed them in *Escherichia coli*. *RparsM* (from *Rhodopseudomonas palustris*) was included for comparison because of the reported high As methylation and volatilization activities.¹⁷ *ArarsM* (940 bp) and *RparsM* (868 bp) fragments were amplified by PCR using Prime STAR HS DNA Polymerase with the primer pairs P3–4 and P5–P6, respectively (Table S2). The PCR amplicons were purified and cloned into *pEASY*-Blunt vector (TransGen) to create plasmids *pEASY*-Blunt-*ArarsM* and *pEASY*-Blunt-*RparsM*. Plasmids were extracted using a Plasmid Mini Kit I (Omega) and digested with *Nde*I and *Xho*I. *ArarsM* and *RparsM* fragments were cloned into plasmid pET29a (+) to create pET29a-*ArarsM* and pET29a-*RparsM*. A pair of recombinant plasmids and the control vector pET29a(+) were transferred to *E. coli* strains AW3110, in which the As-tolerant chromosomal *arsRBC* operon has been deleted, or W3110 (wild-type). The positive clones were verified by DNA sequencing. Arsenic methylation and volatilization by recombinant *E. coli* cells were determined as follows. Cultures were first grown overnight in 100 mL of LB medium with 50 µg mL⁻¹ kanamycin in a shaker at 37 °C. At the late exponential phase, cells were diluted 50-fold into 100 mL of fresh LB medium with 50 µg mL⁻¹ kanamycin, 0.3 µM isopropyl-β-D-thiogalactopyranoside (IPTG), and 10 µM As(III). After incubation for 12 h, both As species in solution samples and the volatile As produced from these cell cultures were analyzed using the methods described above. As(III) resistance assays were performed using recombinant cells of an As(III)-hypersensitive *E. coli* strain AW3110 (DE3) bearing plasmid pET29a-*ArarsM* or pET29a (+) as described previously.¹⁷

Quantitative Real-Time PCR Analysis of *ArarsM* Gene

A single colony of strain SM-1 was incubated in R₂A medium for 3 d until the late exponential phase. The culture was diluted to an OD₆₀₀ of 0.01 into 100 mL of fresh R₂A medium with 0–10 µM As(III) or 0–100 µM As(V) and incubated for 8 h. Cells in 3 mL of culture medium were harvested by centrifugation. Total RNA was extracted using a Bacterial RNA Kit (Omega). The extracted RNA was digested using a TURBO DNA-free Kit (Ambion). cDNA was prepared by reverse-transcription PCR (RT-PCR) using a PrimeScript II first Strand cDNA Synthesis Kit (Takara) with random hexamers. Quantitative real-time PCR was performed by using the cDNA as the template with the primer pair P7–P8 (Table S2) on a CFX96 Thermocycler (Bio-Rad). The *gyrB* gene (GenBank accession no. KU695255) of strain SM-1 was amplified with the primer pair P9–P10 and used as a reference gene.

RESULTS

Changes of As Species in Soil Porewater during Incubation

In an initial experiment, we incubated the Shimen paddy soil under flooded conditions with either air or N₂ in the headspace. Soil porewater was collected periodically for As speciation analysis. As(III) was the predominant As species in the porewater, with As(V) being a minor species under both conditions (Figure S1). The As(III) concentration showed an increasing trend with incubation time and reached a plateau of approximately 0.2 mg l⁻¹ As (2.7 µM) by 30–40 days. DMAs(V) was detected in the porewater samples collected during 30–60 days of incubation with air in the head space, accounting for 14–17% of the total soluble As in the samples. In contrast, no DMAs(V) was detected when the soil was incubated under N₂, suggesting that the microbes mediating As methylation in this soil are not strict anaerobes.

Isolation of a Bacterial Strain with High Rates of As Methylation and Volatilization

To isolate bacteria capable of methylating As in the Shimen paddy soil, we established an enrichment culture using the soil from the surface layer (the top 0.5 cm below standing water) after the soil was incubated for 30 d under flooded conditions with air in the headspace. A total of 23 colonies that grew on the ST10⁻¹ medium were obtained and screened for their As(III)-methylating activity. Only one isolate, designated strain SM-1, was confirmed to have robust As methylation ability. The strain was an aerobic oligotrophic bacterium; it grew better in R₂A medium³⁵ than in ST10⁻¹ medium but could not grow in LB medium.

The time course of transformation of 10 µM As(III) by strain SM-1 in R₂A medium was determined. In the presence of As(III), strain SM-1 produced DMAs(V) and TMAs(V)O rapidly (Figure 1A). The concentration of As(III) decreased by 88.1 ± 0.7% (mean ± SD) during the initial 12 h, with concurrent increases in the concentrations of DMAs(V) and TMAs(V)O in the medium. The concentration of DMAs(V) decreased slightly after 12 h, whereas that of TMAs(V)O remained stable. After 24 h, almost all of the As(III) disappeared from the medium, while DMAs(V), TMAs(V)O, and As(V) accounted for 27.7 ± 1.3%, 23.3 ± 2.1%, and 4.4 ± 0.1%, respectively, of the initial amount of As(III) added.

Volatile As species from the assay was collected by a chemotrap and analyzed by HPLC–ICP-MS (Figure 1B). TMAs(III) was the only volatile As species that was detected as TMAs(V)O due to oxidation by AgNO₃ in the chemotrap. The amount of TMAs(III) produced increased rapidly during the initial 16 h and remained stable between 16 and 24 h. The total amount of trapped volatile As was $35.7 \pm 13.8 \mu\text{g}$, accounting for $47.6 \pm 18.4\%$ of the initial total As. There was an acceptable mass balance of As in the assay system (total recovery $107.8 \pm 20.4\%$), taking into account As in the bacterial cells, which represented $1.2 \pm 0.1\%$ of the total As at 24 h.

Strain SM-1 was also able to produce methylated As species when exposed to $10 \mu\text{M}$ As(V) (Figure 2). However, the presence of a high P concentration (2.1 mM) in the R₂A medium inhibited the uptake of As(V). Estimated from the decrease in medium As(V) concentration, only $25.7 \pm 0.4\%$ of the As(V) was taken up by the bacterial cells after 24 h, of which $5.2 \pm 3.4\%$, $40.8 \pm 1.2\%$, $41.6 \pm 3.0\%$, and $12.5 \pm 1.4\%$ were transformed into As(III), DMAs(V), and TMAs(V)O in the medium and TMAs(III) gas in the headspace, respectively. When the P concentration in R₂A medium was reduced to 0.57 mM , all of the As(V) was taken up by the cells, of which $28.0 \pm 1.8\%$, $56.0 \pm 3.9\%$, and $16.0 \pm 4.6\%$ were transformed into DMAs(V) and TMAs(V)O in the medium and TMAs(III) gas in the headspace, respectively (Figure 2).

Taxonomical Identification of Strain SM-1

Strain SM-1 was a rod-shaped and nonmotile Gram-negative bacterium (Figure S2). Cells of this strain were convex, circular, and orange when grown on the R₂A agar plate for 2 d. Phylogenetic analysis of the 16S rRNA gene sequences (Figure S3) showed that strain SM-1 was most closely related to *Rudanella lutea* DSM 19387^T, with 88% identity. On the basis of the 16S rRNA gene sequence, we identified bacterium SM-1 as a new species in a new genus within the family *Cytophagaceae* and named this species *Arsenicibacter rosenii*.

Effect of *A. rosenii* Strain SM-1 Inoculation on As(III) Methylation in Paddy Soil

We conducted an incubation experiment with soil slurries (Shimen Paddy soil) with or without inoculation of strain SM-1 in combination with or without addition of As(III) for 5 d. A treatment of sterilized soil was also included for comparison. In the sterilized soil, only As(V) and As(III) were detected in the soil porewater, indicating a lack of As methylation (Figure 3A). The presence of the As(V) in this treatment could be due to the abiotic oxidation of As(III) by manganese oxides in the soil. In unsterilized soil without SM-1 inoculation, small amounts of DMAs(V) were detected in the soil porewater. After the addition of As(III) to unsterilized soil, both DMAs(V) and TMAs(V)O were detected. Inoculation of SM-1 into the unsterilized soil greatly increased the DMAs(V) concentration in the porewater, an increase of about 10- and 16-fold in the +As(III) and –As(III) treatments, respectively (Figure 3A). Inoculation of SM-1 also increased the production of TMAs(V)O. The volatile As species TMAs(III) was detected after SM-1 inoculation (Figure 3B).

We used a highly specific primer pair targeting the 16S rRNA gene of *A. rosenii* strain SM-1 bacteria to quantify its abundance in the soil-slurry experiment. The copy number of SM-1

16S rRNA was determined to be approximately 10^6 g⁻¹ soil in the unsterilized soil (Figure 3C). After sterilization, the copy number decreased to approximately 10^4 g⁻¹ soil. In contrast, inoculation of SM-1 into the unsterilized soil increased the copy number of SM-1 16S rRNA to approximately 10^{10} g⁻¹ soil. There was, therefore, a good agreement between the abundance of strain SM-1 and the extent of As methylation and volatilization detected in the different treatments of the soil-slurry experiment.

Cloning and Transcriptional Analysis of *arsM* gene in *A. rosenii* Strain SM-1

On the basis of the draft genome of *A. rosenii* strain SM-1, we identified a putative gene encoding As(III) *S*-adenosylmethionine methyltransferase, designated as *ArarsM*. *ArarsM* is located in an *ars* (arsenic resistance) operon, which includes two other genes, a putative *arsR* gene encoding As(III)-responsive transcriptional repressor, and a putative *arsC* gene encoding As(V) reductase. The open reading frame (ORF) of *ArarsM* is 924 bp long and shows no similarity with any other gene sequences in the NCBI database. The deduced sequence of 307 amino acid residues shares the highest sequence identity (70%) with SIArSM, a putative ArsM homologous protein from *Spirosoma linguale* DSM 74. ArArSM also shares 61%, 44%, 38%, 33%, and 33% identity with the annotated As(III) methyltransferases from *Fibrella aestuarina* (FaArSM), *R. palustris* (RpArSM), *Cyanidioschyzon* sp. isolate 5508 (CmArSM7), *Chlamydomonas reinhardtii* (CrArSM), and human (hAS3MT), respectively. We examined multiple sequence alignments of the deduced amino-acid sequences of the As(III) methyltransferases mentioned above. ArArSM contains four conserved Cys residues at the 30th, 67th, 155th, and 205th positions (indicated by asterisks in Figure S4A) that are characteristic of all ArsM proteins investigated to date and are probably involved in As(III) binding,³⁶ as well as three conserved motifs (underlined in Figure S4A) possibly involved in the interactions with the methyl donor *S*-adenosylmethionine (SAM). Phylogenetic analysis (Figure S4B) showed that ArArSM forms an independent branch with bacterial SIArSM, FaArSM, and RpArSM and is more distant from two algal ArsMs and a mammalian AS3MT. The presence of the putative *arsC* gene could explain the As methylation ability when SM-1 was exposed to As(V) (Figure 2).

Real-time quantitative PCR analysis was performed to investigate whether the expression of *ArarsM* was induced by As(III) or As(V). The transcript level of *ArarsM* increased markedly in response to increasing concentration of As(III) (2–10 μ M) or As(V) (10–100 μ M) (Figure S4C). At 10 μ M As(III) or As(V), the transcript level was 240-fold or 55-fold, respectively, higher than the no-As control. These results show that *ArarsM* is highly inducible by As. The smaller induction by As(V) than As(III) was probably because either As(V) uptake by SM-1 was suppressed by phosphate in the medium or As(V) must be reduced to As(III), which in turn interacts with the transcriptional repressor *ArarsM*.³⁷

Functional Characterization of *ArarsM*

To investigate the function of ArArSM, we cloned the *ArarsM* gene and expressed it in an As(III)-hypersensitive *E. coli* strain AW3110 (DE3), which lacks the As-tolerant chromosomal *arsRBC* operon and has no *arsM* gene,³⁸ as well as in the wild-type *E. coli* strain W3110. For comparison, we also expressed *RparsM* from the soil bacterium *R. palustris* in *E. coli* AW3110. RpArSM exhibits the highest rate of As methylation among all

previously identified ArsMs.³⁴ In LB medium containing 10 μ M As(III), *E. coli* strain AW3110 expressing *ArarsM* transformed almost all As(III) to TMAs(V)O and DMAs(V) in the medium and produced a large amount of volatile TMA(III) in the headspace within 12 h, whereas no As(III) methylation occurred in the control cells (Figure 4A,B and Figure S5A,B). Moreover, *E. coli* AW3110 expressing *ArarsM* produced substantially more methylated As species and volatile As than the cells expressing *RparsM* (Figure 4A,B and Figure S5A,B). Interestingly, when *ArarsM* and *RparsM* genes were expressed in the wild-type strain of *E. coli* (W3110) containing the As(III)-resistant *arsRBC* operon, the *ArarsM* recombinant cells showed greatly diminished activities of As methylation and volatilization, whereas the *RparsM* recombinant cells had no detectable As methylation and volatilization activities (Figure 4A,B and Figure S5C,D).

To test the effect of *ArarsM* expression on As(III) tolerance, we cultured cells of the *E. coli* strain AW3110 expressing *ArarsM* or the vector plasmid in LB medium containing different concentrations of As(III). In the absence of As(III), both the *E. coli* cells bearing the pET29a-*ArarsM* and the vector plasmid showed normal growth, with the former growing slightly faster than the latter (Figure S6). At 50 μ M As(III), cells expressing *ArarsM* grew normally, whereas the control cells exhibited very weak growth. At 70 μ M As(III), growth of the control cells was almost completely inhibited, whereas cells expressing *ArarsM* grew to approximately half of the cell density in the no As(III) treatment. These results suggest that the gene product of *ArarsM* conferred As(III) resistance to *E. coli* strain AW3110.

DISCUSSION

Microbial As methylation and volatilization are important components of the As biogeochemical cycle in the paddy environment.^{13,14} Depending on the geographical region and the growth conditions, rice grain can contain substantial amounts of methylated As species, especially DMAs(V), which probably originate from microbial As methylation in the paddy soil.¹⁵ Despite the importance of As methylation in the paddy environment, little is known about microorganisms mediating this process in paddy soil. To date, only two bacteria (*Streptomyces* sp. strain GSRB54 and a sulfate-reducing bacterium, *Clostridium* sp. BXM) from paddy soils have been characterized with regard to their As methylation activities.^{26,27} In the present study, we identified a novel bacterial strain, SM-1, from a moderately contaminated paddy soil possessing a strong ability to methylate and volatilize As. When cultured in the R₂A liquid medium, the strain transformed nearly all of the As(III) (10 μ M) in the medium into DMAs(V), TMAs(V)O, and volatile TMAs(III) within 24 h, with volatile As accounting for nearly half of the total As (Figure 1). The rate of As methylation appears to be much faster than those reported for *Streptomyces* sp. strain GSRB54 and *Clostridium* sp. BXM, both converting As(III) to MAs(V) and DMAs(V) and producing very little trimethylarsenic compounds.^{26,27} Arsenic volatilization was not quantified in either studies^{27,26} but was determined to likely be insignificant on the basis the mass balance of all As species in the assay medium. Meyer et al.²⁸ reported that an anaerobic bacterial isolate, *Clostridium* sp. strain ASI-1, from an alluvial soil was able to volatilize As into AsH₃, MMAs(III)H₂, DMAs(III)H, and TMAs(III), but the amounts of volatile As were small compared to the amount of inorganic As in the substrate. Therefore,

the ability of strain SM-1 to volatilize As can be considered to be exceptional when compared to other soil bacteria reported in the literature.

Phylogenetic analysis based on 16S rRNA sequences showed that strain SM-1 represents a new species, which we named *A. rosenii*, in a new genus within the family *Cytophagaceae*. Strain SM-1 is an aerobic oligotrophic bacterium and is abundant in the paddy soil from which the strain was isolated, with an estimated 16S rRNA copy number of approximately 10^6 g⁻¹ soil. Inoculation of SM-1 into the paddy soil also greatly enhanced As methylation and the production of volatile TMAs(III) from the soil slurry (Figure 2), indicating a great potential of SM-1 for As bioremediation in contaminated soil.

Because strain SM-1 is an aerobic microorganism, it is likely to occupy the niche of aerobic microzones in flooded paddy soil, such as the soil–water interface and the rhizosphere of rice roots. In these microzones, O₂ is supplied by diffusion through the water layer or released by rice roots through the aerenchyma.³⁹ Indeed, the strain was isolated from a thin soil layer underneath the standing water. When the headspace of the incubation bottles was filled with N₂, no As methylation was observed in the soil (Figure S1), suggesting that aerobic rather than anaerobic microorganisms drive As methylation in the paddy soil studied. The observation that methylated As was detected in the soil porewater only after 30 days of flooded incubation can be explained by the low levels of As(III) during the initial phase of incubation. As(III) was gradually mobilized into soil porewater after soil flooding (Figure S1) as a result of As(V) reduction and the reductive dissolution of iron oxides and hydroxides, which are the main adsorbents of As(V) and As(III).^{9–11} Mobilized As(III) in the porewater of the bulk soil could diffuse to the aerobic microzones and serve as the substrate for As methylation by strain SM-1.

To gain a better insight into the efficient As methylation and volatilization by *A. rosenii* strain SM-1, we cloned the orthologous *arsM* gene (*ArarsM*) and characterized its function. Similar to other As methylating bacteria,¹⁷ *ArarsM* is located in an *ars* operon downstream of *arsR*, which encodes a transcriptional repressor. The expression of *ArarsM* was strongly induced by As(III) and, to a lesser extent, by As(V) (Figure S4), although As(III) is likely to be the true inducer of the gene.¹⁷ The smaller induction by As(V) than As(III) could explain the lower production of volatile TMAs(III) by the strain when exposed to As(V) (Figure 2). When expressed in an As-sensitive strain of *E. coli* with its *ars* operon deleted, *ArarsM* catalyzed methylation of As(III) to DMAs(V) and TMAs(V)O and produced volatile TMAs(III) efficiently (Figure 4). In addition, expression of *ArarsM* conferred As(III) resistance to *E. coli*. The results support the notion that As methylation is a detoxification mechanism in microorganisms.^{17,24,40}

There are several possible reasons for the high efficiency of As methylation and volatilization in *A. rosenii* strain SM-1. First, the expression of *ArarsM* is highly responsive to As(III) (Figure S4). Second, *ArarsM* is highly efficient at catalyzing As methylation reactions from As(III) to TMAs(III) (Figure 4). The efficiency was even higher than *RparsM*, which is known to have a high rate of As methylation, when both were expressed in *E. coli* AW3110 (Figure 4). Third, there are no putative genes encoding As(III) efflux transporters, such as *ArsB* or *Acr3p*, in the draft genome of strain SM-1. The lack of genes

encoding As(III) efflux transporters in strain SM-1 is rather unusual because nearly every prokaryote has either ArsB or Acr3, owing to their importance in As resistance.¹⁶ When strain SM-1 was exposed to As(V), some As(III) was extruded to the external medium (Figure 2), perhaps through passive transporters such as aquaglyceroporin channels (AQPs).⁴¹ However, the extent of As(III) efflux was much smaller than the production of methylated As species, possibly due to the lack of ArsB or Acr3p. This limited As(III) efflux is likely to result in As(III) accumulation in the cell, thus favoring the methylation reactions. The lack of As(III) efflux as a key detoxification mechanism may also impose a strong selective pressure on the microbe, resulting in the evolution of a highly efficient ArArsM. Thus, with a limited As(III) efflux, As methylation and volatilization possibly constitute the key mechanism of As(III) detoxification in strain SM-1. The evidence supporting this hypothesis can be seen in the marked differences in As methylation and volatilization when *ArarsM* or *RparsM* was expressed in *E. coli* strain AW3110 (lacking the As(III) efflux gene) and wild-type strain W3110; As methylation and volatilization diminished greatly in the latter (Figure 4).

In conclusion, we have identified a novel bacterial strain (SM-1) belonging to a new genus of the family *Cytophagaceae* from an As-contaminated paddy soil. This strain is highly efficient at As methylation and volatilization, possibly due to the lack of a strong As(III) efflux mechanism and the highly inducible expression of *ArarsM* encoding an efficient As(III) methyltransferase enzyme. Strain SM-1 is abundant in the paddy soil studied and likely plays an important role in the As biogeochemical cycling. Furthermore, its high ability to volatilize As may have potential applications in the bioremediation of As-contaminated environments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Time course of As(III) transformation by *A. rosenii* SM-1. Arsenic species in culture medium (A) and production of volatile As (trimethylarsine, TMAs(III)) (B) by strain SM-1. Data are means $\pm$ SD ($n = 3$).

Figure 2.

Transformation of As(V) by *A. rosenii* SM-1 after cells were incubated in R₂A and R₂A (low-P) medium with 10 μM As(V) for 24 h. As speciation in culture medium (A) and As volatilization (B) by strain SM-1. Data are means ± SD (*n* = 3).

Figure 3.

Effect of inoculation of *A. rosenii* SM-1 on As methylation and volatilization from Shimen paddy soil after incubation for 5 days. As species in soil porewater (A), the amount of volatile As (B), and copy numbers of strain SM-1's 16S rRNA gene in the soil at the end of the experiment (C). Different letters above bars indicate significant differences ($P < 0.05$). Data are means $\pm$ SD ($n = 4$).

Figure 4.

Transformation of As(III) by *E. coli* As-sensitive mutant AW3110 or wild-type W3110 expressing vector plasmid (pET29a), *ArarsM* (pET29a-*ArarsM*), or *RparsM* (pET29a-*RparsM*). As speciation in culture medium (A) and As volatilization (B) by *E. coli* cells after incubation with 10 μM As(III) for 12 h at 37 °C. Data are means ± SD ($n = 3$).