

Volatilization of Arsenic from Polluted Soil by *Pseudomonas putida* Engineered for Expression of the *arsM* Arsenic(III) S-Adenosine Methyltransferase Gene

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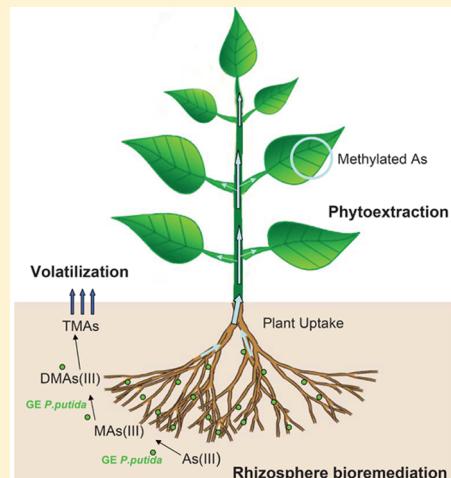
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Supporting Information

ABSTRACT: Even though arsenic is one of the most widespread environmental carcinogens, methods of remediation are still limited. In this report we demonstrate that a strain of *Pseudomonas putida* KT2440 endowed with chromosomal expression of the *arsM* gene encoding the As(III) S-adenosylmethionine (SAM) methyltransferase from *Rhodopseudomonas palustris* to remove arsenic from contaminated soil. We genetically engineered the *P. putida* KT2440 with stable expression of an *arsM-gfp* fusion gene (GE *P. putida*), which was inserted into the bacterial chromosome. GE *P. putida* showed high arsenic methylation and volatilization activity. When exposed to 25 μ M arsenite or arsenate overnight, most inorganic arsenic was methylated to the less toxic methylated arsenicals methylarsenate (MAs(V)), dimethylarsenate (DMA(V)) and trimethylarsine oxide (TMA(V)O). Of total added arsenic, the species were about $62 \pm 2.2\%$ DMA(V), $25 \pm 1.4\%$ MAs(V) and $10 \pm 1.2\%$ TMA(V)O. Volatilized arsenicals were trapped, and the predominant species were dimethylarsine (Me_2AsH) ($21 \pm 1.0\%$) and trimethylarsine (TMA(III)) ($10 \pm 1.2\%$). At later times, more DMA(V) and volatile species were produced. Volatilization of Me_2AsH and TMA(III) from contaminated soil is thus possible with this genetically engineered bacterium and could be instrumental as an agent for reducing the inorganic arsenic content of soil and agricultural products.



INTRODUCTION

Arsenic is a ubiquitous metalloid¹ that is introduced into the environment through both geological and anthropogenic sources, such as mining activities, utilization of arsenic-based pesticides or herbicides.^{2–4} Arsenic in soils and sediments enters food chain from edible parts of vegetables or cereals that have accumulated high levels of arsenic and poses a serious health risk to humans. Inorganic arsenic has been classified as class one carcinogen by the International Agency for Research on Cancer.⁵ Long-term exposure has resulted in a global epidemic of arsenic-related diseases, with many people having developed skin lesions, kidney, lung, and bladder cancers.^{6,7} There is an urgent need to efficiently remove arsenic from contaminated water and soils.

In situ bioremediation of arsenic by microorganisms has been widely hailed because of their potential advantages in facilitating cost-effective and environmental friendly technolo-

gies.^{8–10} Bacteria containing the arsenic(III) S-adenosylmethionine methyltransferase (*arsM*) gene were able to sequentially methylate toxic inorganic arsenic^{11,12} to less toxic pentavalent methylated arsenicals such as methylarsenate (MAs(V)), dimethylarsenate (DMA(V)) and trimethylarsine oxide (TMA(V)O). Whether biomethylation of arsenic was considered as a detoxification process is still controversial.¹³ The intermediate metabolites methylarsonous acid (MAs(III)) and dimethylarsinous acid (DMA(III)) are generated during this process. These trivalent methylated arsenical intermediates are highly toxic and possibly carcinogenic, but they can be easily oxidized to much less toxic MAs(V) and DMA(V), so

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they are not readily detected in environmental settings. Biovolatilization of the final product, gaseous trimethylarsine (TMAs(III)), could remove arsenicals from polluted sites,¹⁴ while converting the extremely toxic inorganic species of arsenic to virtually innocuous counterparts, thereby adding to the potential toolbox for bioremediation of soil arsenic.¹⁵

Bioremediation of arsenic from contaminated soils using genetically engineered (GE) bacteria might be a better option as the amount of volatile arsenic generated by indigenous microorganisms is limited. Recently, Liu, et al.¹⁶ successfully overexpressed an *arsM* gene in *Sphingomonas desiccabilis* and *Bacillus idriensis* and improved arsenic volatilization from contaminated soil. *Pseudomonas putida* KT2440, an efficient colonizer of the root system of various plants, is a ubiquitous saprophytic bacterium endowed with a remarkable adaptability to diverse environments, including soils heavily contaminated with multiple metals.¹⁷ This soil microbe has been studied extensively as a model organism for biodegradation of aromatic compounds^{18–20} and it has been highlighted as an optimal chassis for implantation of synthetic genetic circuits.²¹ *P. putida* KT2440 has two systems for arsenic tolerance based on arsenate reduction to arsenite and ensuing export of arsenite to the external medium, a feature that affords endurance to very high concentrations of the corresponding anions.²² However, *P. putida* does not have an *arsM* gene and cannot methylate arsenic. We previously reported the construction of GE *P. putida* expressing the *Chlamydomonas reinhardtii arsM* gene (*CrarsM*).²³ This transgenic microbe exhibited high levels of arsenic methylation. Recently *arsM* genes from a variety of microorganisms have been identified and shown to exhibit quite variable capacity for arsenic methylation.^{11,24} The construction of new GE bacteria with higher arsenic methylation become possible by selecting increasingly active products of *arsM* genes. One problem is that plasmids carrying exogenous genes (*arsM* genes) are often lost during practical application. We can solve this dilemma by inserting the cloned genes into the chromosome rather than expressing from a plasmid. This also increases the stability and safety of the strain, and reduces the likelihood of horizontal gene transfer.

In this study we also chose *P. putida* KT2440 as the host strain for arsenic removal. The *Rhodopseudomonas palustris arsM* (*RparsM*) gene was selected because of its high rate of methylation. The *RparsM* gene was inserted into the chromosome of *P. putida* KT2440 using a synthetic transposon vector that allows random generation of fusions between a promoterless *arsM* gene and external transcription initiation signals, thereby allowing selection for different degrees of arsenic methylation and resistance. The selected genetically engineered construct exhibited a high level of arsenic methylation and volatilization compared to wild type. In the environmental arsenic-contaminated soil assay, GE *P. putida* exhibited a high capacity for arsenic volatilization, which is 9-fold higher than wild type cells. Further experiments for rhizosphere bioremediation by plants and GE *P. putida* will be conducted. To monitor colonization of GE *P. putida* in soil, the *RparsM* gene was fused with the gene for green fluorescent protein (*gfp*), and the fusion gene was stably integrated into the chromosome of *P. putida* KT2440. The GE *P. putida* exhibits a high level of fluorescence when exposed to arsenic. These fluorescent bacteria can be visualized in situ in the environment and in the rhizosphere.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, Medium, and Reagents. The *Pseudomonas putida* KT2440 strains, *E. coli* CC118λpir, *E. coli* HB101 (pRK600) and pBAM1 plasmid used in this study have been described before.²⁵ *E. coli* strain DH5α (Promega, Madison, WI) was used for plasmids construction, replication. Strain AW3110(DE3) (Δ *arsRBC*),¹² which is hypersensitive to arsenic(III), was used for complementation studies. Strains BL21(DE3) (Invitrogen) was used for protein expression. *E. coli* cells were grown aerobically in Luria–Bertani (LB) medium reference (10 mL) at 37 °C. *P. putida* was cultured at 30 °C. Selection of *P. putida* cells was made onto M9 minimal medium plates²⁵ with citrate (2 g/L) as the sole carbon source. Antibiotics, when needed, were added at the following final concentration: ampicillin (Ap) 100 µg/mL for *E. coli* and 500 µg/mL for *P. putida*, kanamycin (Km) 50 µg/mL and chloramphenicol (Cm) 30 µg/mL for both species. To construct a vector for *RparsM* transfer, the *RparsM* gene was retrieved from plasmid pET28. Bacterial growth was monitored by measuring the optical density at 600 nm (OD₆₀₀). All reagents were obtained from commercial sources.

Plasmid Construction. For expressing *RparsM* of *R. palustris* in *P. putida* KT2440, plasmid pBAM1-*RparsM*, in which *RparsM* was promoterless and has the sequence of a C-terminal six-histidine tag, was constructed. *RparsM* under control of T₇ promoter and with a C-terminal six-histidine was cloned from plasmid pET28a-*RparsM*.¹² The promoterless *arsM* with a C-terminal His tag was removed by double digestion with *SacI* and *XbaI* and ligated to pBAM1 which was digested with the same restriction enzymes. A *gfp* reporter plasmid was constructed to form pBAM1-*RparsM-gfp*, in which the *gfp* was fused to the C-terminal of *RparsM* to replace of six-histidine tag. *RparsM* was cloned from pET28a-*RparsM* using primers: 5'- GCGAGCTCAAGGAGATATACCATGGC -3' (*SacI* site underlined) and 5'- GCTCTAGAATGATGCCCG-GAGGAGCG -3' (*XbaI* site underlined). The PCR fragment was gel purified and digested with restriction enzymes underlined. The *gfp* gene was obtained from pGreen vector, which was digested by *XbaI*. The two fragments were ligated into vector plasmid pBAM1 in sequence. pBAM1 was digested with the same restriction enzymes, generating plasmid pBAM1-*RparsM-gfp*.

Expression of RparsM in *P. putida* KT2440. Plasmid pBAM1 and its derivatives were delivered from *E. coli* CC118λpir (pBAM1 or pBAM1-*RparsM-gfp*) donor cells into *P. putida* KT2440 by tripartite mating with the assistance of the helper strain *E. coli* HB101 (pRK600).²¹ The conjugation mixture was incubated at 30 °C for 6 to 8 h by tripartite mating on membrane filters (0.45 µm, Millipore) on LB agar plates. The filter was transferred to 5 mL of 10 mM MgSO₄ and vortexed to suspend the cells. Afterward, appropriate dilutions were plated onto selective medium as indicated for counter-selecting against the donor cells. The conjugation mixture was plated on minimal selective medium as indicated. *P. putida* KT2440 containing different expression vectors were cultured overnight in LB medium at 30 °C. Cells were harvested and identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Western blot analysis was used to probe the expression of *RparsM*. Proteins separated by SDS PAGE were transferred to a nitrocellulose membrane (Protran, Schleicher & Schuell). Western blot analysis was performed

using a Western Lighting Ultra chemiluminescence substrate kit (PerkinElmer) using an antimouse IgG to the six histidine tag.

Resistance Assays to Arsenic. The arsenic resistance phenotype of GE *P. putida* KT2440 cells was determined in bacteria. Cells of wild type bacteria (bearing pBAM1), and bacteria bearing pBAM1-RparsM, which were used for inorganic arsenic sensitivity assays, were grown overnight in M9 medium²⁶ at 30 °C, supplemented with containing 25 µg/mL kanamycin and 0.2% glucose. Overnight cultures were adjusted to 1.0 OD₆₀₀, diluted in a 10-fold series with double distilled water, and spotted (2 µL) onto M9 solid medium supplemented with the indicated concentrations of arsenite and antibiotics.²³ Growth was scored after incubation at 30 °C for 24 h. The growth rate of the two strains in liquid medium was also monitored. After growing in LB liquid medium to log phase (OD₆₀₀ 0.6–0.8), the cells were harvested by centrifugation and suspended in M9 medium. Cells were then inoculated to a final OD₆₀₀ of 0.004–0.006. The growth of the cultures was monitored over 24 h and the OD₆₀₀ was plotted as a function of growth time.

Arsenic Speciation in the Culture Medium. To investigate the effects of arsenic methylation and volatilization by wild type and GE *P. putida* (expressing RparsM) in the M9 medium, arsenic methylation by bacteria was performed in capped 20 mL vials with a total volume of 4 mL and sodium arsenite was added to each vial in a final concentration of 25 µM. The vials were incubated for 16 h at 30 °C. Each treatment was triplicate. The supernatant were filtered and stored at 4 °C until analysis. To trap volatile arsenicals, 2 cm nitrocellulose membrane filters were put in vial caps and impregnated with 0.15 mL of 6% H₂O₂.¹¹ The filters were digested with 0.2 mL of 70% HNO₃ at 70 °C for 20 min. The digestion solutions were diluted 25-fold and stored in 4 °C. Arsenic speciation analysis was conducted within 24 h by HPLC-ICP-MS (Series 2000, PerkinElmer) with anion exchange column.

Arsenic Volatilization from Arsenic-Contaminated Soil. Soil was sampled from farmland near a mining company at Zhuzhou, Hunan province, China. Soil was air-dried and sieved with 2 mm mesh. Soil pH was 5.82, organic matter was 3% and total arsenic was 20.0 mg/kg. The experimental device was described as reported.²⁷ Briefly each Erlenmeyer flask (250 mL) contained unsterilized soil (40 g) with water holding capacity at 30%, (unflooded). The wild type or GE *P. putida* KT2440 were cultured in the LB medium at exponential phase, and this culture medium (2 mL) was inoculated into flasks containing soil. The control (without strain inoculation) was supplemented with an equal volume of LB medium. Trapping tubes for volatile arsenic were prepared by filling the silica gel beads impregnated with 10% AgNO₃ into glass tubes and connected to the flask. The headspace was refreshed by pumping filtered air with pumps at intervals (24 h) for 10 min each time. In order to increase bioavailable arsenic in the soil to investigate the effect of arsenic bioavailability on arsenic volatilization, arsenite were spiked into subsamples to a final concentration of 30 mg/kg. Each treatment was replicated for five times. All flasks were shaken in the dark at 150 rpm at 30 °C for 6 days. All trapping tubes were taken off, and samples were prepared as described²³ and stored at 4 °C until analysis.

Determination of Arsenic Species and Total Arsenic. The concentrations of total arsenic were measured by ICP-MS. Arsenic speciation in the LB medium was determined by HPLC-ICP-MS.¹² Briefly, either a reverse-phase C18 column (Jupiter 300) was eluted isocratically with a mobile phase

consisting of 3 mM malonic acid, 5 mM tetrabutylammonium hydroxide, and 5% methanol, pH 5.6, with a flow rate of 1.0 mL/min or an anion-exchange column (PRP X100, Hamilton) was eluted with a step gradient of mobile phase A (20 mM ammonium bicarbonate, pH 8.5) and mobile phase B (20 mM ammonium bicarbonate, pH 7.0) at a flow rate of 1.5 mL/min. Indium (10 µg/L) was added online postcolumn as the internal standard. Retention times for arsenic species were determined using a mixture of standards comprised of 1 µM each of As(III), As(V), DMA(V), MA(V), and MA(III).

Imaging Bacteria. After 6 days inoculation of bacteria in the soil, soil samples (0.5 g) were washed with sterilized water (5 mL). Subsamples of supernatant (100 µL) were inoculated in LB plates supplemented with kanamycin (50 µg/mL) to inhibit growth of indigenous bacteria and stored at 30 °C for 48 h. Photographs were taken in visible and UV light.

RESULTS AND DISCUSSION

Selection of arsM Genes for Expression in *P. putida* KT2440. Arsenite methylation is clearly a detoxification mechanism,^{11,12} and widespread in nature.²⁸ Methylation has been observed in members of many kingdom.²⁸ Many orthologous arsM encoding putative orthologs of As(III) methyltransferases have been reported, including RparsM (*Rhodopseudomonas palustris* CGA009),¹² hAS3MT (*Homo sapiens*)²⁹ NsarsM (*Nostoc sp.* PCC7120),²³ CrarsM (*Chlamydomonas reinhardtii*),²² and CmarsM (*Cyanidioschyzon* sp. SS08).¹¹ To choose a suitable arsM gene for high arsenic methylation and volatilization in *P. putida* KT2440, a comparison was made for all the arsMs arsenic methylation activity. RparsM, CrarsM and NsarsM were shown to confer a high arsenic(III) resistance when expressed in an arsenic-sensitive strain of *E. coli* AW3110.¹² CmarsM and hAS3MT showed a relatively low arsenic(III) resistance (Supporting Information (SI), Figure S1A, B). Further arsenic speciation analysis showed that each arsM examined could convert As(III) to DMA(V) or TMA(V)O after 12 h of incubation (Figure 1A). TMA(III) and some Me₂AsH were the volatile products, both were easily oxidized to TMA(V)O and DMA(V), respectively (Figure 1B). Among these arsMs, RparsM exhibited the highest rate of arsenic volatilization. The intracellular arsenic species of *E. coli* AW3110 expressing various arsMs was quantified (Figure 1C). RparsM produced substantial amounts of TMA(V)O (88%). CrarsM also showed a high methylation activity with DMA(V) (50%) and TMA(V)O (43%). CmarsM shows a least activity due to the incubation temperature is not the optimal one (60–70 °C).¹¹ To remove arsenic from soil, RparsM was the best for arsenic volatilization by genetically engineered *P. putida* KT2440. To obtain optimum expression of foreign RparsM gene in the *P. putida* KT2440 strain, we compared the codon usages of RparsM to *P. putida*. Fortunately the triplet codons for the RparsM are on their relative abundance in the *P. putida* genome. More frequent codons of *P. putida* are preferred in RparsM gene. RparsM gene was chosen for the further experiments without any code optimization.

Chromosomal Expression of RparsM and Arsenic Resistance of GE *P. putida* KT2440. The chromosomal engineering RparsM to *P. putida* KT2440 is a prerequisite to realize the full potential of this species in the arsenic detoxification and bioremediation. RparsM was cloned into the transposon vector pBAM1 (SI, Figure S2) and transferred from *E. coli* cc118λpir to *P. putida* KT2440 by tripartite

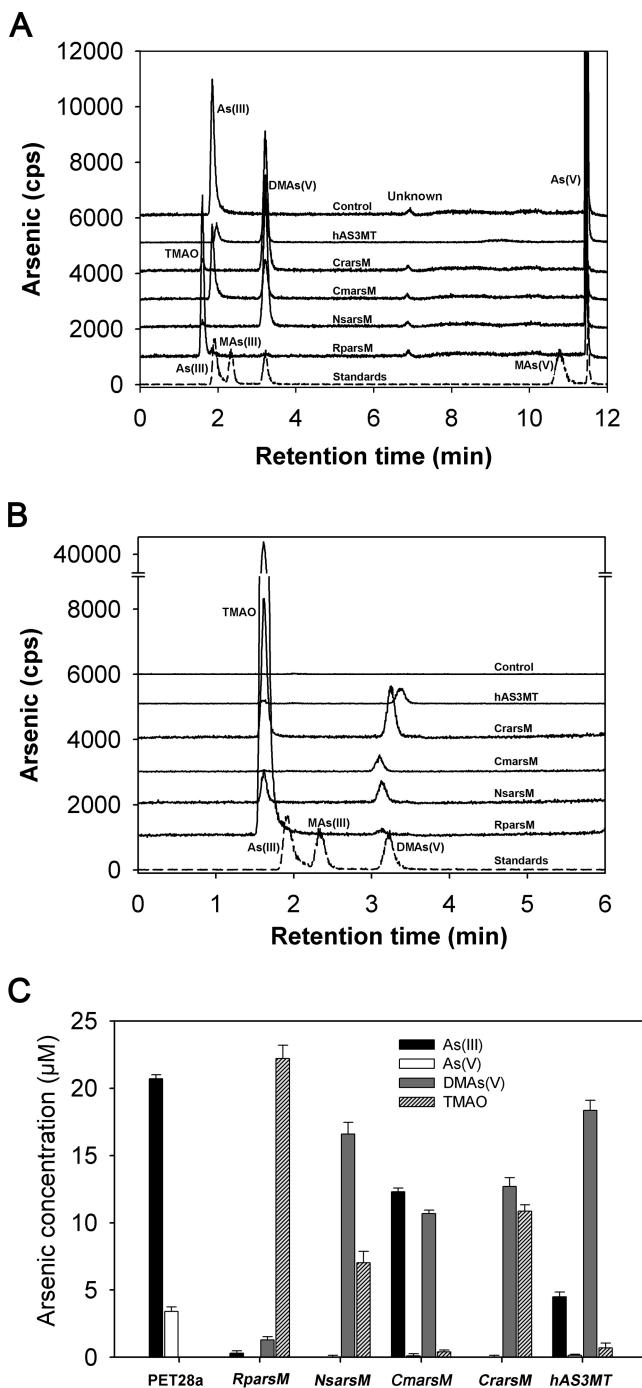


Figure 1. Formation of methylated arsenicals was measured in cells of *E. coli* AW3110 (DE3) (Δ arsRBC), bearing vector plasmid pET28a(+) as a control; RparsM (*Rhodopseudomonas palustris* CGA009); NsarsM (*Nostoc* sp. PCC 7120); CmarsM (*Cyanidioschyzon* sp. 5508); CrarsM (*Chlamydomonas reinhardtii*); hAS3MT (*Homo sapiens*). A, Speciation of arsenic in the culture medium by different *arsM* genes; B, Volatilization of arsenic by *E. coli* AW3110 expressing different *arsM* genes. Cells were incubated for 12 h in LB medium with 25 μ M As(III). C, arsenic speciation in the culture medium. Cells were grown for 12 h. Soluble arsenic species were determined by HPLC-ICP-MS using an anion-exchange column. Cps, counts per second.

conjugation. To examine whether exconjugants had undergone authentic transposition events or resulted from the cointegration of pBAM1-RparsM into the host genome, 100 colonies were randomly selected, and their sensitivity to the plasmid

marker (Ap^R) was examined. All 100 Km^R clones were sensitive to ampicillin (500 μ g/mL), thereby indicating that the insertion of the minitransposon carried by pBAM1 occurred as expected. Several colonies were resistant to As(III), indicating that the promoterless RparsM had been inserted into chromosomal genes in the correct orientation and in the proper reading frame. It was easier to screen the positive transformants for RparsM fused with *gfp*, where fluorescent colonies could be chosen for the further analysis. *P. putida* KT2440 is a ubiquitous saprophytic bacterium endowed with a remarkable adaptability to diverse environments including tolerance to different heavy metals and metalloids. Two copies of an *arsRBCH* operons exist in the chromosome of *P. putida* KT2440¹⁹ allowing it to grow in the presence of 2 mM As(III). One of the *ars* operon have three additional genes located downstream of *arsH*. The putative functions of these genes products are proposed to be a phosphatase, a monooxygenase, and a phosphinothricin N-acetyltransferase respectively, but their relationship to arsenic resistance is unclear. Neither *ars* operon has an *arsM* gene, so wild type *P. putida* is unable to methylate As(III). When exposed to 7.5 mM As(III), cells expressing RparsM grew dramatically better than those bearing the empty vector (Figure 2A). In addition, cells bearing vector plasmid pBAM1 were sensitive to 10 mM As(III). In contrast, cells expressing RparsM could grow in As(III) concentration as high as 10 mM (Figure 2A), demonstrating that the gene products confer tolerance to As(III). Furthermore, the growth curve in liquid M9 medium were also consistent with the arsenic resistance results observed on solid medium (SI, Figure S3). Genetically engineered *P. putida* KT2440 exhibits much more resistance to As(III), which can be used for in situ remediation of arsenic-polluted soil. It also shows a high capacity for arsenic volatilization both in culture and in environmentally simulated arsenic-contaminated soil. Therefore, practical application of GE *P. putida* for arsenic biotransformation from arsenic contaminated soils is becoming possible.

Arsenic Methylation by GE *P. putida* KT2440. The amount of volatilized arsenic was proportional to the loss of arsenic from the medium (Figure 2B). GE *P. putida* biomethylated As(III) primarily to DMA(V), with some TMA(V)O, after 12 h of incubation (SI, Figure S4A and B). Wild type *P. putida* cells exhibited resistance to As(III) due to its two *ars* operons, no methylated or volatilized arsenicals were detectable. The amount of volatile TMA(III) increased with time (Figure 2B and SI, Figure S4B). After 48 h, Me₂AsH (21 ± 1.0%) and TMA(III) (10 ± 1.2%) were the volatile arsenic species. The arsenic species detected in the medium were 62 ± 2.2% DMA(V), 25 ± 1.4% MA(V) and 10 ± 1.1% TMA(V) O of total arsenic (Figure 2C). Due to the existence of two copies of *arsC* in the chromosome of *P. putida* KT2440, As(V) is rapidly reduced to As(III). We have shown that the initial product of arsenic methylation is MA(III), and it is likely that the second product is DMA(III),³⁰ but these are rapidly oxidized to the pentavalent species in soil,³¹ so only MA(V) and DMA(V) can be detected. To representative of soil conditions, low phosphate medium and M9 medium were also used to measure the GE *P. putida* KT2440 methylation activity. As(V) was totally converted to methylated arsenic by GE *P. putida* similar to the rate of methylation of As(III) in both LB medium and a low phosphate medium (data not shown). In M9 medium, the arsenic methylation and volatilization were not efficient due to the high phosphate concentration in this

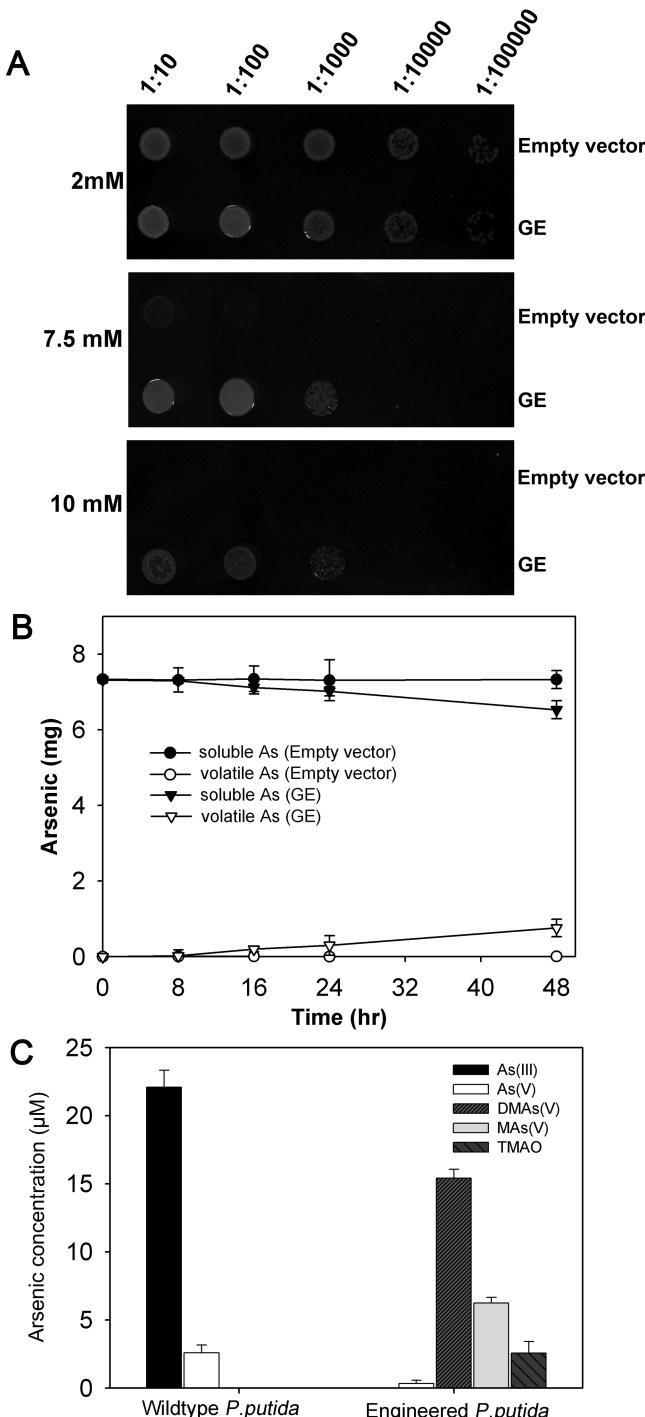


Figure 2. In vivo formation of methylated arsenicals by GE *P. putida* KT2440. A, Chromosomally expressed *RparsM* enhances arsenic resistance in *P. putida* KT2440. Growth of cells in LB medium plates with 2, 7.5, and 10 mM As(III). B, Transformation of medium arsenic into volatile species. Soluble (solid symbols) and volatile (open symbols) arsenic species formed by *E. coli* strain BL21 (DE3) with vector plasmid pET28a (circles) or pET28a-*RparsM* (inverted triangles) in solution (solid symbols) were determined following growth in LB medium in the presence of 25 μM As(III) for 16 h. Total arsenic in the culture medium and cells (black) and volatilized arsenic (white) were determined by ICP-MS, as described above. Data are the mean \pm SE ($n = 3$). C, arsenic speciation in the culture medium. Cells were grown for 24 h. Soluble arsenic species were determined by HPLC-ICP-MS using an anion-exchange column. Cps, counts per second.

medium, which inhibited the uptake of arsenate by cells. These results demonstrated that heterologous expression of *RparsM* in the chromosomal of *P. putida* conferred the ability to methylate and volatilize arsenic. It efficiently decreased the levels of inorganic arsenic and converted it to volatile arsenic species.

The main product of methylation by the genetically modified microbe is DMA_n(V). Like As(III), the protonated, uncharged forms of the methylated As species MA_n(V) and DMA_n(V) enter rice roots through the aquaporin channel OsLsi1.³² In contrast, MA_n(V) and DMA_n(V) are not complexed by thiols, which is very mobile and more efficiently translocated from roots to the shoot in rice.^{33,34} DMA_n(V) can be removed from soil by plants by uptake through nodulin 26-like intrinsic (NIP) aquaporin channels and accumulated in plant tissues. *P. putida* is a robust and versatile rhizosphere bacterium. The establishment of *P. putida* on plant roots is a rapid and dynamic process.²⁰ Many plant species accumulate methylated arsenicals such as MA_n(V), DMA_n(V), and TMA_n(V) if they are available in soil.^{35,36} Thus, using plant-rhizosphere bacterium symbiosis to clean up the arsenic by harvesting plants is a promising and potentially practical method for bioremediation. GE *P. putida* with *RparsM* has the prospect for efficiently removing arsenic from contaminated soil by a combination of rhizosphere biovolatilization and phytoextraction by host plants. The increased resistance to arsenic by the engineered rhizobacteria *P. putida* at higher concentrations is particularly important, as it could provide a competitive advantage to the GE *P. putida* in a contaminated soil environment. This could be crucial factor for sustaining the growth of the engineered strain in the presence of the native bacterial population. As a rhizosphere bacterium, *P. putida* can colonize and persist in the root system of a number of plants. This is a promising concept for rhizosphere bioremediation and phytoextraction.

Expression of *RparsM* in GE *P. putida* KT2440. To elucidate the mechanism of arsenic methylation by the GE *P. putida*, the protein expression level of *RparsM* (29.7 kDa) in GE *P. putida* was determined. Compared to *RparsM* expressed in *E. coli* (BL21) behind the *T₇* promoter, expression of *RparsM* was low in GE *P. putida*. It was expected that expression from chromosome would be less than from a multicopy plasmid. There was no difference from wild type with or without As(III) induction (SI, Figure S5A). *RparsM* expression was probed by Western blotting with antibody against the six histidine tag. *RparsM* expressed in BL21 (DE3) under control of the *T₇* promoter was used as positive control. A band at 30 kDa corresponding to the predicted size of *RparsM* was detected in GE *P. putida* (SI, Figure S5B). Although chromosomal expression of *RparsM* in *P. putida* KT2440 was lower than on a plasmid under the *T₇* promoter in *E. coli*, high level of expression might constitute a selective disadvantage to the organism, particularly when competing with indigenous organisms.³⁷ Microbes that carry multiple copies of foreign genes or express those genes at a high level often compete poorly with indigenous bacteria in their natural environment. Plasmids are often not maintained in genetically engineered bacteria in the absence of selective pressure and are lost during attempts at practical application. In genetically modified microorganisms, it is frequently preferable in some circumstances to insert the cloned genes into the chromosome, where they are maintained at a low, natural copy number and thus, at least theoretically, they should be as stable as chromosomal genes.³⁸ Microorganisms bearing chromosomal inserts with the genes of interest have advantages to compete

with indigenous organisms than their counterparts with plasmids. In our previous study, an *arsM* gene from the eukaryotic unicellular alga *Chlamydomonas reinhardtii* was inserted into the *P. putida* chromosome under control of the kanamycin promoter.²³ Compared to that construct, our present one in which the *arsM* gene from the bacterium *R. palustris* is chromosomally inserted showed a considerably higher capacity for arsenic methylation and volatilization.

The GE *P. putida* was Monitored by Fluorescence. To monitor growth of *P. putida* under environmental conditions, we constructed plasmid pBAM1-*RparsM-gfp*, in which green fluorescent protein was fused to the C-terminus of RparsM in place of the six histidine tag, allowing direct fluorescent detection of hybrid protein production rather than just mRNA synthesis. The exconjugant clones were screened on M9 agar plates for green fluorescent colonies by illuminating the plate with UV light, and fluorescent colonies were screened for methylation activity (Figure 3). Use of a *gfp* tag allows for facile selection of recombinants with high methylation activity. Only the colonies with high fluorescence were picked up for further experiments, making this time-consuming screening much easier. In addition, *P. putida*, as a root-colonizing bacterium, are very relevant in phytoremediation. It is important to understand the effect of *P. putida* as inoculator and whether they cause changes in the microbial community structure in the rhizosphere and soil. Using this strategy, it conferred the GE *P. putida* have fluorescence and the process of these labeled strains colonizing all surfaces of plant root zones, such as roots hairs and lateral roots can be easily monitored, including the effect of inoculation on soil microbial populations.

Arsenic Volatilization from Soil by GE *P. putida* KT2440. To examine the ability of GE *P. putida* KT2440 to volatilize arsenic in environmental soil samples, cells were inoculated in unsterilized soil, and volatilization of arsenic was quantified. In comparison with soil not inoculated with bacteria, wild type of *P. putida* KT2440 did not show increased arsenic volatilization (Figure 4). In contrast, GE *P. putida* exhibited a high capacity for arsenic volatilization from arsenic-contaminated soil. The amount of volatile arsenic was about 9-fold of that of wild type cells, although volatilization from soil was less than from LB medium, which may reflect lower arsenic bioavailability in soil. Arsenite was amended into the soil to increase its bioaccessibility. In the control (soil + As(III)) and wild type (soil + WT + As(III)), there are no significant difference in arsenic volatilization compared with unamended soil. However, addition of As(III) significantly increased arsenic volatilization by GE *P. putida* KT2440. The amount of volatilized arsenic was 49-fold that of unamended soil with the GE cells, indicating that arsenic bioaccessibility is a major limiting step in arsenic removal via arsenic volatilization. Since bioavailability of As in contaminated soils is an important factor determining the remediation efficiency, future studies should also be directed to manipulating As bioavailability, such as the amendment of soil organic matter and soil water management. Under UV light it could be seen that there are many more fluorescent cells with GE *P. putida* inoculation (SI, Figure S6B) than with wild type inoculation (SI, Figure S6A), even though growth of both looked similar under visible light (SI, Figure S6A, B). This result indicates that GE *P. putida* KT2440 grow better in arsenic-contaminated soil as arsenic is methylated and volatilized.

In future experiments, arsenic removal from contaminated soil by GE *P. putida* KT2440 expressing either *RparM* or

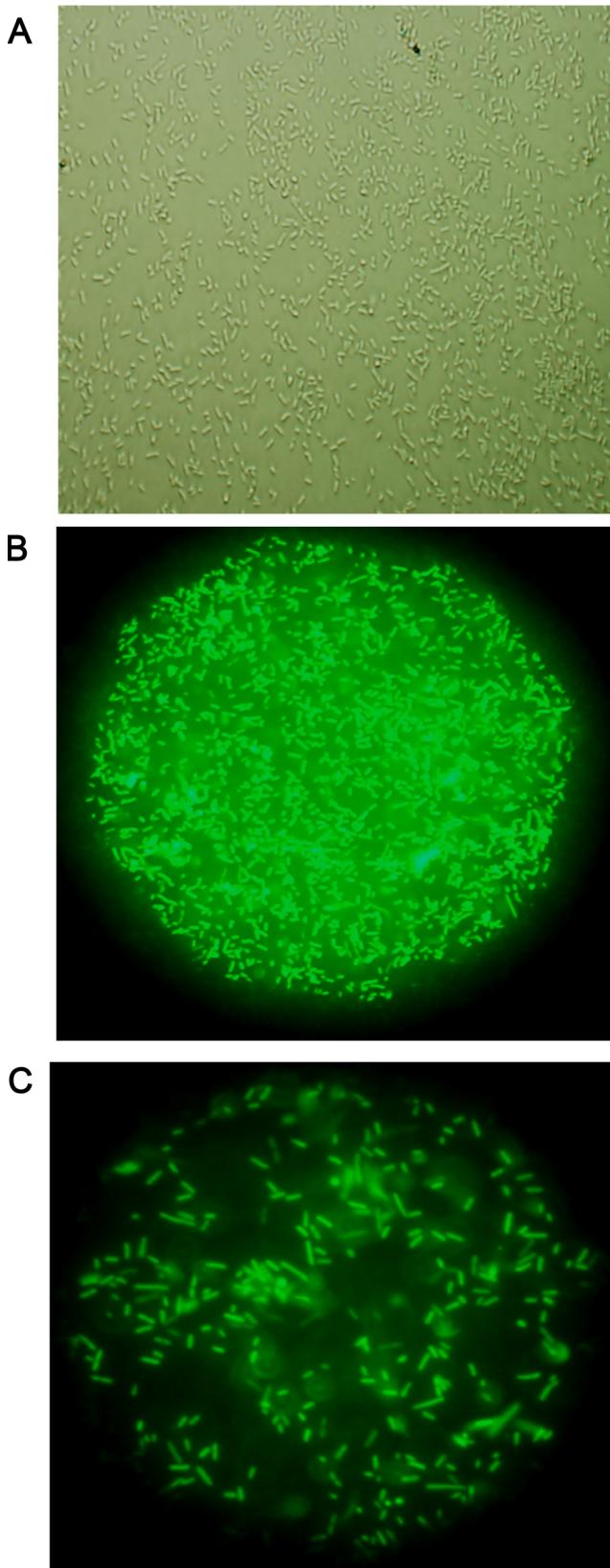


Figure 3. Fluorescence of GE *P. putida* expressing the *RparsM-gfp* fusion. (A), Growth of the cells was examined by epifluorescence microscopy under white light. (B), Growth of the cells was examined by epifluorescence microscopy under UV light. (C), Higher magnification images from (B).

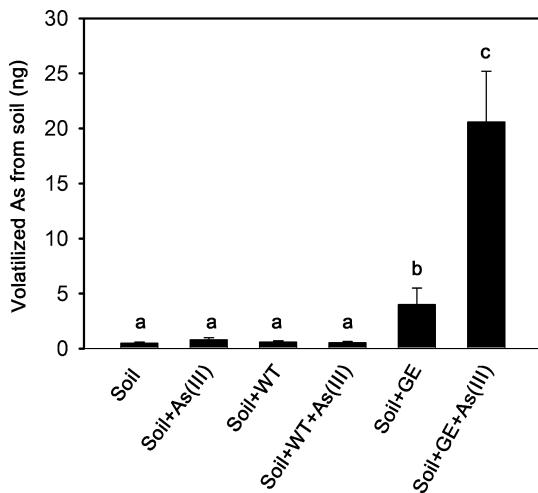


Figure 4. Amount of volatilized arsenic by wild type and GE *P. putida* KT2440 in arsenic contaminated soil (20 mg/kg) and contaminated soil amended with As(III) (10 mg/kg). The value was mean \pm SE and $n = 3$. The different letters above bars indicate significant differences ($P < 0.005$).

RparsM-gfp in combination with plants will be examined. The rhizosphere is the most active portion in soil, which has important ecological implications for soil function, including biogeochemical cycles. Furthermore, a wider range of soil types, soil properties and soil with different arsenic contamination levels will be further utilized to evaluate the efficiency of GE *P. putida* in arsenic removal. The relative toxicity of an arsenical depends primarily on the species, whether it is inorganic or organic, its oxidation state and solubility. Inorganic trivalent arsenite has high toxicity and high mobility, but mineral-bound arsenicals such as sulfide forms are highly insoluble, so the efficiency for GE *P. putida* to remove these arsenic compounds need to be further evaluated. A better understanding of these processes is critical for maintaining the safety of our food supply.^{39,40} A genetically engineered microbe that exhibits fluorescence when it methylates arsenic will be especially useful for monitoring this critical biogeochemical process at the plant root-soil interface. Our results suggest the potential feasibility of using symbiosis between GE *P. putida* chromosomal expressing RparsM and plant as an efficient strategy for arsenic remediation from contaminated soil. Although more toxic MAs(III) and DMAAs(III) are generated during this methylation pathway, both are rapidly methylated to the volatile end product TMAs(V)O. In addition, they are chemically unstable and are quickly oxidized to the nontoxic pentavalent forms. This process is promising for harnessing the root system of plants to increase yields of staple food crops.

Of concern for any new bioremediation scheme is whether it is better and safer than the methods that it replaces. Large-scale interventions on arsenic-contaminated soil based on the GE strain described in this paper must be preceded by a thorough evidence-based risk assessment study to ensure safety to the surrounding animal, plant, and human landscape. In this context, the question of the toxicity and fate of TMAs(III) is key to diagnose the applicability of such an strategy. Bartolomeo Gasio (1863–1944) demonstrated that fungi grown on arsenic produced a gas that could kill rats.⁴¹ This “Gosio Gas” was thought to be TMAs(III). However, recently Cullen has called this an “urban myth” and has showed that TMAs(III) is entirely nontoxic.⁴² Inhalation by animals or

humans in the target site would be expected to result in rapid elimination in urine, resulting in a negligible hazard. If produced by soil bacteria during the day, the half-life of atmospheric TMAs(III) can be measured in minutes or 8 h⁴³ under daytime conditions. However, at night when there is no photodegradation, TMAs(III) is stable for days, providing sufficient time for the gas to be diluted into the “infinite sink” of the atmosphere. Subsequent photooxidation would ensure dispersed to areas of low arsenic. These hypothetical scenarios should be endowed with solid evidence and quantitative parameters before large-scale applications can be contemplated in earnest. While such studies are beyond the scope of this paper, we advocate our approach, that is, engineering soil microbes for arsenic methylation and volatilization as a valuable and worth considering avenue for tackling the phenomenal challenge of this type of environmental pollution.

ASSOCIATED CONTENT

Supporting Information

Figures S1–S6. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

- As(V) arsenate
- As(III) arsenite
- MAs(V) methylarsenate
- DMAAs(III) dimethylarsenate
- Me₂AsH dimethylarsine
- DMAAs(V) dimethylarsenate
- TMAs(III) Trimethylarsine
- TMAs(V)O trimethylarsine oxide
- HPLC high performance liquid chromatography
- ICP-MS inductively coupled mass spectroscopy
- SDS PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis

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