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Arsenic methylation by a genetically engineered *Rhizobium*-legume symbiont

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Abstract

Background and aims—Arsenic (As) is one of the most widespread environmental contaminants. The aim of our study was to test a novel bioremediation system based on the symbiosis between leguminous plant and genetically engineered rhizobia.

Methods—The arsenite [As(III)] *S*-adenosylmethionine methyltransferase gene (*CrarsM*) from the alga *Chlamydomonas reinhardtii* was inserted into the chromosome of *Rhizobium*

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leguminosarum bv. *trifolii* strain R3. The As methylation ability of the recombinant *Rhizobium* was tested under free living conditions and in symbiosis with red clover plants. Arsenic speciation was determined using high-performance liquid chromatography-inductively coupled plasma mass spectrometry.

Results—Under free-living conditions, *CrarsM*-recombinant *R. leguminosarum* gained the ability to methylate As(III) to methylated arsenicals, including methylarsenate [MAs(V)], dimethylarsenate [DMAs(V)] and trimethylarsine oxide [TMAs(V)O]. Red clover plants were inoculated with either control (non-recombinant) or *CrarsM*-recombinant *R. leguminosarum* and exposed to 5 or 10 μ M arsenite. No methylated As species were detected in red clover plants inoculated with control *R. leguminosarum*. In contrast, all three methylated species were detected in both the nodules and the shoots when the recombinant *Rhizobium* established symbiosis with red clover, accounting for 74.7–75.1% and 29.1–42.4% of the total As in the two plant tissues, respectively. The recombinant symbiont also volatilized small amounts of As.

Conclusions—The present study demonstrates that engineered rhizobia expressing an algal *arsM* gene can methylate and volatilize As, providing a proof of concept for potential future use of legume-rhizobia symbionts for As bioremediation.

Keywords

Arsenic; Arsenite *S*-adenosylmethionine methyltransferase; Arsenic methylation; Bioremediation; Rhizobia; Symbiosis

Introduction

Arsenic (As) is a ubiquitous toxic metalloid that is released into the environment from both anthropogenic and geochemical sources (Smith et al. 1998). Arsenic contamination has become a major public health concern world-wide. In Asia, millions of people are exposed to unsafe levels of As in drinking water (Fendorf et al. 2010; Meharg 2004; Rodriguez-Lado et al. 2013). Soil contamination of As is also common in these regions due to mining, smelting, irrigation of As-laden groundwater and use of As-containing agrochemicals (Williams et al. 2006; Zhu et al. 2008). In addition to drinking water, humans are also exposed to As via dietary intake, with rice being the most important dietary source of inorganic As (Li et al. 2009; Meharg and Zhao 2012). There is a need to develop methods to remediate contaminated soils and to reduce the transfer of As from soil to the food chain (Zhao et al. 2010).

Contaminated soils may be cleaned up with the aid of microorganisms or plants, processes termed bioremediation and phytoremediation, respectively. A number of attempts have been made to use plants and associated microorganisms to remove contaminants from soils (Pilon-Smits 2005). For example, microbial genes encoding mercuric reductase (MerA) and organomercurial lyase (MerB) have been engineered into plants to transform highly toxic ionic mercury (Hg^{2+}) or methyl-mercury to less toxic and volatile elemental mercury (Hg^0) (Bizily et al. 2000; Rugh et al. 1998). In another study, the gene for the bacterial arsenate reductase (ArsC) was engineered into *Arabidopsis thaliana* plants, and the resulting plants became As tolerant and accumulated high amounts of As (Dhankher et al. 2002). In addition

to oxidation or reduction of As, some microorganisms are able to methylate inorganic As into organic species, some of which are volatile (Bentley and Chasteen 2002). Arsenic methylation is catalyzed by the enzymes encoded by the As(III) *S*-adenosylmethionine methyltransferase (*arsM*) genes (Qin et al. 2006). In contrast, higher plants do not have the ability to methylate inorganic As (Lomax et al. 2012). Microbial *ArsM* enzymes sequentially methylate inorganic As to mono-, di- and tri-methyl arsenical compounds, with the final product trimethylarsine [TMAs(III)] being volatile (Bentley and Chasteen 2002; Qin et al. 2009; Ye et al. 2014). Microbial As(III) methylation can potentially be applied to bioremediation by transforming toxic As(III) to less toxic TMAs(III) gas (Cullen and Bentley 2005). However, natural As volatilization from soil is limited (Mestrot et al. 2011), suggesting that indigenous soil microbes that volatilize As are either not abundant or inactive. To enhance As methylation, *arsM* genes can be transferred into a host that has a high survival capability in specific soil compartments. In a previous report, Chen et al. (2013) genetically engineered *Pseudomonas putida* KT2440 with the alga *Chlamydomonas reinhardtii* *arsM* gene (*CrarsM*) and showed that the engineered bacterium could methylate and volatilize As. Huang et al. (2015) overexpressed an *arsM* gene from the thermophilic alga *Cyanidioschyzon merolae* in *Bacillus subtilis* 168 and showed that the engineered bacterium could volatilize As from As-contaminated compost. Transgenic rice expressing a bacterial *arsM* gene from the bacterium *Rhodospseudomonas palustris* showed enhanced As volatilization, although the overall efficiency of As methylation was very low (Meng et al. 2011). In a recent study, the algal *CrarsM* gene was expressed in *Arabidopsis thaliana*, and the transgenic plants gained the ability to methylate and volatilize As, but appeared to suffer from DMAs(V) toxicity (Tang et al. 2016b).

In the present study, we investigated a new approach for As bioremediation by using symbiosis of a legume plant with recombinant *Rhizobium* expressing an algal *arsM* gene. Rhizobia are Gram-negative bacteria that can establish a symbiotic relationship with leguminous plants, and undergo differentiation into endo-symbiotic forms known as bacteroids. Bacteroids can fix nitrogen in the nodules as ammonia for assimilation by the host plant (Mylona et al. 1995). The use of the legume-rhizobia symbiont in bioremediation has the advantage of overcoming the potential limitation of microbial colonization in the rhizosphere. Here we demonstrate that the engineered *Rhizobium* can methylate arsenite [As(III)] both in free living conditions and in symbiosis with legume plants.

Materials and methods

Bacterial strains and plant materials

Rhizobium strain R3 is a spontaneous streptomycin-resistant (Sm^r) mutant of *Rhizobium leguminosarum* bv. *trifolii* strain ACCC18001 obtained from Agricultural Culture Collection of China (ACCC). No *arsM* gene could be cloned from this strain. Mini-transposon delivery plasmid pBAM1 was used as a suicide vector to generate stable integrants (Martinez-Garcia et al. 2011). *Escherichia coli* S17-1 λ pir (Tp^r Sm^r *recA* *thi* *pro* *hsdR*⁻ M^- RP4-2-Tc:Mu:Km Tn7) was used to introduce plasmids into *Rhizobium* strain R3 by conjugation (Delorenzo et al. 1990). Unless otherwise indicated, *E. coli* strains were cultured at 37 °C in Luria-Bertani

(LB) broth (Sambrook and Russel 2011), and *Rhizobium* cells were grown at 28 °C in liquid TY medium or on TY-agar 1.5% (wt/vol) plates (Beringer 1974).

Plasmid construction and transformation of *R. leguminosarum*

The *CrarsM* gene was amplified from the alga *C. reinhardtii* cDNA library with a primer pair (forward: 5'-GGAATTCCATATGGTGGAGCCGGCTTCCATCGCGGAGCTT-3', *NdeI* site underlined) and (reverse: 5'-CCGCTCGAGTTAATGATGATGATGATGGCAGCAGGCGCCGCCGGGG-3', *XhoI* site underlined). *CrarsM* expression under the control of *Km* promoter and fused with a C-terminal six-histidine was placed into the plasmid pBAM1, which formed plasmid pBAM1-P_{km}-*CrarsM*-*his* as described by Chen et al. (2013). To investigate the expression of *CrarsM*, the green fluorescent protein gene (*gfp*) was fused to the C-terminal of *CrarsM*. The *gfp* gene was cloned from pGreen vector with an *EcoRI* site incorporated into both the 5' and 3' end (Chen et al. 2014). After *EcoRI* digestion, the fragment was cloned into the *EcoRI* site of pBAM1-P_{km}-*CrarsM*, generating the plasmid pBAM1-P_{km}-*CrarsM*-*gfp*, which was subsequently transferred into *E. coli* S17-1λ*pir* by electro-transformation. The *CrarsM*-*gfp* fusion gene was transferred into the chromosome of *Rhizobium* strain R3 by conjugation with *E. coli* S17-1λ*pir* using the protocol described by Lozano et al. (2013). The conjugation mixture was incubated at 28 °C for 12 h on membrane filters (0.45 µm, Millipore) on TY-agar plates. Selection of transconjugants was performed on TY-agar plates supplemented with streptomycin (50 µg mL⁻¹) and kanamycin (50 µg mL⁻¹). Several clones were isolated and further confirmed by polymerase chain reaction (PCR) and restriction digestion.

Arsenic tolerance and transformation assays

To determine As(III) resistance of the recombinant *Rhizobium* strains R3(pBAM1) and R3(pBAM1-P_{km}-*CrarsM*-*gfp*), 0.5 mL of a stationary phase 24 h culture of either strain was inoculated into 50 mL of liquid TY medium containing different As(III) (NaAsO₂) concentrations (0, 50, 100, and 300 µM). The cultures were incubated at 28 °C with shaking at 180 rpm. Samples were taken every 12 h during a 72 h period. Cell density was measured at 600 nm using a UV-vis spectrophotometer (Shimadzu UV 1800).

Arsenic methylation in free-living rhizobia was investigated. R3(pBAM1) or R3(pBAM1-P_{km}-*CrarsM*-*gfp*) cells were inoculated into 50 mL of liquid TY medium supplemented with 10 µM As(III). Each strain was replicated in three flasks. The cultures were incubated at 28 °C with shaking at 180 rpm. Aliquots were taken every 12 h during a 72 h period for As speciation analysis. Culture samples were centrifuged at 10,000×*g* for 5 min, and the supernatants were filtered through 0.22 µm membrane filters. The supernatants were analyzed for As speciation directly or after H₂O₂ (10%, v/v) oxidation to separate TMAs(V)O from the co-eluting As(III). The remaining cells were collected by centrifugation, and the cell pellets were digested for total As determination by ICP-MS. Volatile methylarsines released from the culture were trapped using an AgNO₃ impregnated silica gel described previously (Mestrot et al. 2009).

Arsenic transformation in red clover-recombinant *Rhizobium* symbiont

Red clover plants were inoculated and grown as described previously (Lomax et al. 2012). Red clover seeds were sterilized with 1% NaOCl for 10 min, washed thoroughly with sterile water and germinated in darkness at 30 °C for 3 days. Germinated seedlings were immersed in the culture broth of either *Rhizobium* strain R3(pBAM1) or R3(pBAM1-P_{km}-*CrarsM-gfp*) containing approximately 10⁴ cells mL⁻¹ for 1 h. One seedling was planted aseptically in each boiling tube containing sterile one quarter-strength Hewitt's nutrient solution without nitrogen (Hewitt 1966) and different concentrations of As(III) (0, 5, or 10 µM), solidified with 1.5% agar. Each As(III) treatment was replicated in 12 boiling tubes. The tubes were covered with a sterile breathable sealing film and placed in a green-house with controlled temperatures (day/night: 25 °C /16 °C) and light intensity (250 µmol m⁻² s⁻¹).

After 6 weeks, plants were removed from agar tubes, washed thoroughly with tap water and then with deionized water. Plants were separated into nodules, roots and shoots. Nodules on each plant were counted, quickly frozen in liquid nitrogen and stored at -70 °C for further analysis. The fresh weights of shoots and roots were determined. Roots were submerged in an ice-cold desorption solution (1 mM K₂HPO₄, 0.5 mM Ca(NO₃)₂ and 5 mM MES, pH 6.0) for 15 min with periodic shaking to remove apoplastic As (Xu et al. 2007). Samples were ground in liquid nitrogen using a pestle and mortar. To extract As species, a phosphate buffer solution (2 mM NaH₂PO₄ and 0.2 mM Na₂-EDTA, pH 6.0) was added to the finely ground shoot and root samples, sonicated for 1 h and then centrifuged at 10,000×*g* for 10 min at 4 °C (Xu et al. 2007). The supernatants were filtered through 0.22 µm membrane filters for As speciation analysis using HPLC-ICP-MS. Aliquots of plant samples were dried at 70 °C, homogenized and digested with concentrated H₂SO₄-H₂O₂. Total N content in the digest was determined using a continuous flow analytical system (3-AA3 Auto Analyzer). Four replicates each were used for total N analysis, As speciation analysis and RNA extraction, respectively.

To monitor *CrarsM* gene expression in bacteroids, nodules from plants inoculated with strain R3(pBAM1-P_{km}-*CrarsM-gfp*) were cut open by hand and placed on a glass slide with a drop of sterile water. A cover slide was placed on the specimen. The fluorescence of the GFP-tagged cells was visualized at the excitation wave-length of 488 nm using a confocal laser scanning microscope (Carl Zeiss LSM780).

Analysis of *CrarsM* gene expression by reverse transcription PCR (RT-PCR)

Total RNA was isolated from fresh culture (OD_{600nm} ≈ 0.6) of strain R3(pBAM1) or R3(pBAM1-P_{km}-*CrarsM-gfp*) using RNA extraction and purification kits (Transgen, Beijing, China). To assess whether *CrarsM* was expressed in the bacteroids, total RNA was isolated from frozen nodule samples using RNeasy Plant Mini kit (Qiagen). The extracted total RNA was reverse transcribed as cDNA with a TaKaRa reverse transcription kit (TaKaRa, Dalian, China). Semi-quantitative RT-PCR was performed using a primer pair (forward: 5'-ATGCCCACTGACATGCAAGAC-3' and reverse: 5'-TCACCCGCAGCAGCGCGCCG-3') targeting *CrarsM* gene as described by Tang et al. (2016b). The DNA gyrase subunit B (*gyrB*) gene was used as the internal reference gene and

amplified using a primer pair (forward: 5'-GCTGTCCGTCTGGTTGAA-3' and reverse: 5'-CGCTGAGGGATGTTGTTGG-3').

Measurement of volatile As

Arsenic species volatilized by strain R3(pBAM1-P_{km}-*CrarsM-gfp*) were trapped as described by Mestrot et al. (2009). Volatile arsenicals released from red clover plants were trapped according to the method of Meng et al. (2011). Silica gel (0.5 mm diameter) was soaked in 5% HNO₃ overnight, washed with Millipore deionized water, impregnated with 10% AgNO₃ solution (w/v) overnight, and then dried at 70 °C. The AgNO₃ impregnated silica gel was loaded into a 3 mL burette and held in by a small quantity of quartz wool at each end. The trap tube was covered with aluminum foil to avoid photodecomposition of AgNO₃. In the assay with strain R3(pBAM1-P_{km}-*CrarsM-gfp*), the *Rhizobium* culture was kept in a 250 mL Erlenmeyer flask connected to a glass joint with an inlet of air from an air pump (ACO-008; 2 W power) and an outlet connected to a silica gel trap tube. The trapping period lasted for 72 h for *Rhizobium* strains, and one week for red clover plants. On the 3rd and 7th days, the silica gel tubes were removed for analysis. Arsenic species trapped by the silica gel were eluted in 1% HNO₃ using a microwave digestion system (CEM Microwave Technology Ltd., Matthews, NC, USA). The working program was as follows: 55 °C for 10 min, 75 °C for 10 min, and 95 °C for 30 min, with 5 min ramp time between each stage. The supernatants were filtered through 0.22 µm filters prior to As species analysis.

Arsenic speciation analysis

Arsenic speciation was determined using high performance liquid chromatography coupled to inductively coupled plasma mass spectrometry (HPLC-ICP-MS, Perkin Elmer NexION 300X, USA) as described previously (Liu et al. 2010). Arsenic species were separated using an anion exchange column (Hamilton PRP X-100, 250 mm length). A solution containing 8.5 mM NH₄H₂PO₄ and 8.5 mM NH₄NO₃ (pH 6.0) was used as the mobile phase, which was pumped through the column isocratically at a flow rate of 1 mL min⁻¹. Indium was added to the post-column solution and measured by ICP-MS as the internal standard. ICP-MS was set up in the He gas collision mode to minimize polyatomic interferences on *m/z* 75 (As). Peaks of arsenite [As(III)], arsenate [As(V)], monomethylarsonate [MAs(V)], dimethylarsenate [DMAs(V)] and trimethylarsenic oxide [TMAs(V)O] were identified and quantified by comparing their retention times with those of the standards.

Statistical analysis

The significance of the treatment effects was assessed by analysis of variance (ANOVA), followed by comparisons between treatment means using the least significant difference (LSD) at *P* < 0.05. All statistical analyses were performed using SPSS 18.0 software (SPSS Inc., Chicago, Illinois, USA).

Results

Expression of *CrarsM* in free-living *Rhizobium* cells

Semi-quantitative RT-PCR showed that *CrarsM* was expressed in recombinant R3(pBAM1-P_{km}-*CrarsM-gfp*) cells (Supplementary Fig. S1A), whereas no *CrarsM* transcript was

observed in the cells of the control strain R3(pBAM1). Microcolonies and individual cells of R3(pBAM1-*P_{km}-CrarsM-gfp*) produced green fluorescence (Supplementary Fig. S1B), indicating that *CrarsM* was active in strain R3(pBAM1-*P_{km}-CrarsM-gfp*).

To determine whether expression of *CrarsM* increased As tolerance, cultures of R3(pBAM1) and R3(pBAM1-*P_{km}-CrarsM-gfp*) were grown in the presence of various concentrations of As(III). Growth of R3(pBAM1-*P_{km}-CrarsM-gfp*) was essentially identical to that of the control strain R3(pBAM1) in the absence of As (Fig. 1), suggesting that the exogenous *CrarsM* gene does not suppress the growth of rhizobia under free-living conditions. Compared with strain R3(pBAM1), cells of R3(pBAM1-*P_{km}-CrarsM-gfp*) grew better in the presence of 50–300 μ M As(III) (Fig. 1). At 300 μ M As(III), growth of the control strain R3(pBAM1) was completely inhibited, whereas R3(pBAM1-*P_{km}-CrarsM-gfp*) was still able to grow.

As(III) methylation and volatilization by recombinant rhizobia

As(III) methylation by strain R3(pBAM1-*P_{km}-CrarsM-gfp*) in TY medium with 10 μ M As(III) was determined over 72 h. DMAs(V) was the predominant methylated species in the medium (Fig. 2a). The concentration of DMAs(V) increased rapidly between 12 and 48 h, concomitant with a decrease in the concentration of As(III). Small amounts of MAs(V) and TMAs(V)O were also detected. At 72 h, most of the inorganic As(III) had been transformed into DMAs(V) ($78.8 \pm 1.3\%$ of the total As) and TMAs(V)O ($8.6 \pm 0.5\%$ of the total As) (Fig. 2a). At 72 h, the bacterial cells contained only 1.2% of the total As added to the culture medium. The total recovery of As in the assay system was 108%. In contrast, no methylated As species were detected in the medium containing the control strain R3(pBAM1) (Fig. 2b).

At 72 h, DMAs(V) and TMAs(V)O were detected in the volatile As trap tubes. These two As species were the oxidized products from the reaction of the volatile DMAs(III)H and TMAs(III) with AgNO₃, respectively. The amounts of TMAs(III) and DMAs(III)H volatilized were 409 and 171 ng, respectively (Fig. 2c), together accounting for 1.5% of the total As(III) added to the assay medium. The control strain R3(pBAM1) produced no detectable volatile As.

Plant growth and expression of *CrarsM* in bacteroids

Red clover plants inoculated with either strain R3(pBAM1) or R3(pBAM1-*P_{km}-CrarsM-gfp*) were grown on agar without or with 5 or 10 μ M As(III). Because nitrogen was not added to the growth medium, the plants relied on N₂ fixation by the nodules. ANOVA showed no significant difference in the number of nodules per plant between the vector control and the recombinant *Rhizobium* with *CrarsM*, whereas 10 μ M As(III) significantly ($P < 0.01$) decreased the nodule number in plants infected with both inoculants (Supplementary Fig. S2A). Shoot and root dry weights of red clover were higher in plants inoculated with R3(pBAM1-*P_{km}-CrarsM-gfp*) than those with R3(pBAM1), but the differences did not reach the $P < 0.05$ significance level ($P = 0.09$ and 0.08 for shoot and root dry weight, respectively) (Supplementary Fig. S2B). The treatment with 10 μ M As(III) decreased shoot dry weight, but the effect also did not reach the $P < 0.05$ significance level ($P = 0.08$). Nitrogen concentration in red clover shoots was significantly ($P < 0.05$) lower in plants

inoculated with R3(pBAM1- P_{km} -*CrarsM-gfp*) than those with R3(pBAM1) (Supplementary Fig. S2C). However, this difference was probably caused by a larger shoot bio-mass in the former, because there was no significant difference in the total amount of N between the two treatments. Arsenic treatment had no significant effect on N concentrations or contents in shoots or roots.

The *CrarsM* gene transcript was detected in bacteroids containing R3(pBAM1- P_{km} -*CrarsM-gfp*), but not in the R3(pBAM1) control (Fig. 3A). Nodules were also examined under confocal laser scanning microscope. Green fluorescence was observed inside the nodules infected with R3(pBAM1- P_{km} -*CrarsM-gfp*) (Fig. 3B) but not in those infected with the control strain (data not shown). These results indicate that both the *CrarsM* and *gfp* genes were expressed under the control of the *Km* promoter within the bacteroids.

Arsenic speciation in red clover

Arsenic speciation was analyzed in shoots, roots and nodules of red clover infected with either R3(pBAM1) or R3(pBAM1- P_{km} -*CrarsM-gfp*). Plants were grown for 6 weeks in agar culture with 5 or 10 μ M As(III). In plants infected with the control strain, no methylated arsenicals were detected in plant tissues, with As(III) being the predominant As species and As(V) being a minor species (Fig. 4). Importantly, substantial amounts of methylated As species were found in the shoots and nodules of red clover infected with R3(pBAM1- P_{km} -*CrarsM-gfp*). In the 5 μ M As(III) treatment, MAs(V), DMAs(V) and TMAs(V)O accounted for 1.2%, 34.4% and 6.7%, respectively, of the total As in the shoots, and 18.8%, 50.5% and 5.9%, respectively, of the total As in the nodules. No methylated As was detected in the roots (excluding nodules). There was no significant difference in the total As concentration in the shoots, roots or nodules between plants infected with R3(pBAM1- P_{km} -*CrarsM-gfp*) or the control strain. A similar pattern of As speciation was obtained in plants exposed to 10 μ M As(III) (Fig. 4). MAs(V), DMAs(V) and TMAs(V)O accounted for 0.9%, 21.4% and 6.7%, respectively, of the total As in the shoots infected with strain R3(pBAM1- P_{km} -*CrarsM-gfp*). The corresponding percentages in the nodules were 18.8%, 51.2% and 4.7%, respectively. Infection with strain R3(pBAM1- P_{km} -*CrarsM-gfp*) resulted in a significant ($P < 0.05$) decrease in the total As concentration in the roots, but an increase, though not significant ($P = 0.10$), in the total As concentration in nodules.

To quantify As volatilization, volatile arsenicals emitted from the red clover plants infected by strain R3(pBAM1- P_{km} -*CrarsM-gfp*) were collected over 7 days. In the 5 and 10 μ M As(III) treatments, the amounts of volatile arsenicals were 2.0 ± 0.1 and 5.7 ± 1.0 ng plant⁻¹ respectively (Fig. 5), accounting for 0.01–0.02% of the total As in the plants. While this may not be high, it is significant that no volatile arsenicals were detectable in the control plants.

Discussion

Rhizoremediation, harnessing the remediation potential of plant roots and the associated rhizosphere microorganisms, is one of the phytoremediation technologies in soil bioremediation. One of the primary limitations associated with this technology is that microbial strains used for rhizoremediation are usually not good rhizosphere colonizers, thus hindering the efficiency of bioremediation. An alternative approach is to introduce

functional genes into bacteria that are good colonizers in the rhizosphere (Rahman et al. 2014; Zhuang et al. 2007).

In this study, we tested the idea of using legumerhizobia symbionts for As bioremediation by introducing an algal As(III) methyltransferase gene (*CrarsM*) into *Rhizobium leguminosarum* bv. *trifolii*, which lacks an endogenous *arsM* gene. The engineered cells gained the ability to methylate inorganic As, transforming nearly all of the inorganic As in the culture medium into methylated As species. DMAs(V) was the main product of As(III) methylation, with MAs(V) and TMAs(V)O as the minor species. The engineered *Rhizobium* strain expressing *CrarsM* also produced volatile As species with TMAs(III) and DMAs(III)H being the major and minor volatile arsenicals, respectively, although together they accounted for only a small percentage of the total As(III) added to the medium.

Engineered *Rhizobium* strain R3 was able to methylate As in symbiosis with red clover without impacting N₂ fixation or plant growth. The most important finding in this study is that the majority of As in the nodules and considerable proportions of As in red clover shoots were methylated As species, with DMAs(V) being the predominant species (Fig. 4). In contrast, red clover plants infected with the control strain of *Rhizobium* did not methylate arsenic. This is in agreement with Lomax et al. (2012), who found that red clover was not able to methylate As. Surprisingly, no methylated As species were detected in the root tissues of red clover infected with the engineered *Rhizobium*. DMAs(V) is highly mobile during the root to shoot translocation (Li et al. 2009; Raab et al. 2007; Tang et al. 2016a; Ye et al. 2010), so it is possible that methylated As species produced in the nodules are efficiently transported to the above ground tissues with little retention in roots. For example, Tang et al. (2016b) showed that transgenic *A. thaliana* expressing *CrarsM* contained most DMAs(V) in the shoots but very little DMAs(V) in the roots, suggesting that DMAs(V) had been efficiently transported to the shoots.

Despite substantial As methylation in the nodules, very small amounts of volatile arsenicals were produced by red clover and the engineered *Rhizobium* symbiont (Fig. 5). This result suggests that the first and second methylation steps catalyzed by CrArsM are very fast, while the third methylation step is very slow. Thus DMAs(V) builds up and is only slowly methylated a third time to the volatiles species TMAs(III). This interpretation is supported by the observation that *Chlamydomonas reinhardtii* has only limited ability to volatilize As (Tang et al. 2016b). The efficiency of As methylation and volatilization varies widely among different microorganisms (Qin et al. 2009; Qin et al. 2006; Wang et al. 2014; Zhang et al. 2015), possibly due to differences in microbial growth and the catalytic ability of ArsM enzymes.

Arsenic methylation is a detoxification mechanism in microorganisms (Qin et al. 2006; Zhang et al. 2015), probably because microbes are able to get rid of methylated As products through volatilization or extrusion to the external medium. In the present study, expressing *CrarsM* in *Rhizobium* also enhanced its tolerance to As under free-living conditions (Fig. 1b). In contrast, DMAs(V) is more toxic than inorganic As in higher plants, perhaps because DMAs(V) cannot be detoxified via the pathway of thiol complexation and subsequent vacuolar sequestration, which is the main detoxification mechanism for inorganic As (Tang

et al. 2016a, 2016b). On the other hand, DMAs(V) is much less toxic than inorganic As in animals, so alternate explanations for DMAs(V) toxicity in plants cannot be excluded (Zhao et al. 2013). For example, trivalent DMAs(III) may be produced during the As methylation process, which could be more toxic to plants than DMAs(V). Whether DMAs(III) existed in the engineered symbiont remains unknown. The need to use H₂O₂ oxidation to separate TMAs(III) from As(III) during As speciation analysis precluded the detection of DMAs(III), if any, in the plant samples. In this study, red clover plants infected by engineered *Rhizobium* showed no significant differences from the control plants in the nodule number or the plant biomass in the treatments with 5 or 10 µM As(III) (Supplemental Fig. S2), suggesting that plants did not suffer from the toxicity of DMAs(V) generated by the recombinant *Rhizobium*. This may be because the concentration of DMAs(V) in the nodules or shoots never achieved toxic levels.

To our knowledge, the present study is the first attempt to use legume and genetically modified *Rhizobium* as a bioreactor for As bioremediation. The results provide a proof of concept, demonstrating that engineered *Rhizobium* expressing *CrarsM* methylate As in nodules and alter As speciation in the host plants. However, As volatilization by the symbiont was limited. The catalytic ability of ArsM enzymes to convert DMAs(V) to volatile TMAs(III) is likely to be a limiting factor for achieving efficient bioremediation. Future studies should explore the As volatilization potential of various microbial *arsM* genes in order to increase the bioremediation efficiency in a legume - engineered *Rhizobium* symbiont. For example, a recent study showed the *arsM* from a soil bacterium *Arsenicibacter rosenii* encodes a highly efficient enzyme for As methylation and volatilization (Huang et al. 2016). This gene could be tested in further studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.
Growth curve of *Rhizobium leguminosarum* strains in TY medium with or without As(III). Data represent means $\pm$ SD ($n = 3$). R3(pBAM1) (vector control); strain R3(pBAM1-P_{km}-CrarsM-gfp) expressing *CrarsM* under the control of the *Km* promoter

Fig. 2.

Arsenite methylation by *Rhizobium leguminosarum* strains R3(pBAM1- P_{km} -*CrarsM-gfp*) (a) and R3(pBAM1)(vector control) (b), and arsenic volatilization by strain R3(pBAM1- P_{km} -*CrarsM-gfp*) (c). The strains were cultured in TY medium with 10 μM As(III) at 28 °C for 72 h. No methylated As species was detectable in the vector control. Data represent means $\pm$ SD ($n = 3$). As(III), arsenite; As(V), arsenate; MAs(V), methylarsenate; DMAs(V), dimethylarsenate; TMA(V)O, trimethylarsine oxide

Fig. 3.

Expression of *CrarsM* in bacteroids colonizing the nodules of red clover plants infected with engineered rhizobia R3(pBAM1-*P_{kmr}*-*CrarsM-gfp*) (GE:R3) and wild-type R3(pBAM1) (WT) determined by semi-quantitative RT-PCR (**A**) and localization of CrarsM-GFP in bacteroids colonizing the root nodules shown by green fluorescence under confocal microscope (**B**). *a*, representative microscopy image of a cut nodule expressing the CrarsM-GFP fusion protein; *b*, bright-field image; *c*, overlap of the GFP and bright-field images

Fig. 4. Effects of engineered *Rhizobium leguminosarum* strains on As(III) methylation in red clover nodules, roots and shoots. Plants were infected with strain R3(pBAM1-P_{km}-CrarsM-gfp) or R3(pBAM1) (vector control), and exposed to 5 μ M (left panels) or 10 μ M (right panels) As(III) concentrations. Data are means $\pm$ SE ($n = 4$). Different letters above bars indicate significant difference ($P < 0.05$) in the total As concentration

Fig. 5. Arsenic volatilization from red clover inoculated with *Rhizobium leguminosarum* strain R3(pBAM1-P_{km}-*CrarsM-gfp*). (A) HPLC-ICP-MS chromatograms of volatile arsenic species (oxidized products by AgNO₃ in the chemotrap). Line a, standards containing TMAs(V)O and DMAs(V); line b, red clover plant infected with control strain R3(pBAM1) exposed to 5 μM As(III); line c, red clover plant infected by strain R3(pBAM1-P_{km}-*CrarsM-gfp*) exposed to 5 μM As(III); line d, red clover plant infected by strain R3(pBAM1-P_{km}-*CrarsM-gfp*) exposed to 10 μM As(III). (B) The amount of volatile arsenic emitted by red clover plants infected with strain R3(pBAM1-P_{km}-*CrarsM-gfp*). Data are means ± SE (*n* = 4)