Arsenic detoxification and evolution of trimethylarsine gas by a microbial arsenite *S*-adenosylmethionine methyltransferase

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In this article, a mechanism of arsenite [As(III)] resistance through methylation and subsequent volatization is described. Heterologous expression of *arsM* from *Rhodopseudomonas palustris* was shown to confer As(III) resistance to an arsenic-sensitive strain of *Escherichia coli*. ArsM catalyzes the formation of a number of methylated intermediates from As(III), with trimethylarsine as the end product. The net result is loss of arsenic, from both the medium and the cells. Because ArsM homologues are widespread in nature, this microbial-mediated transformation is proposed to have an important impact on the global arsenic cycle.

As(III) | ArsM | methylation

As genomes are sequenced, it is becoming clear that nearly all bacteria and archaea have arsenic-resistance (*ars*) operons that confer resistance to arsenite [As(III)] and arsenate [As(V)] (1). The widespread occurrence of *ars* genes reflects the fact that arsenic is a ubiquitous environmental toxic metal. In most cases, these operons encode transport proteins that extrude As(III) from cells. In eukaryotes, As(III) detoxification involves glutathionylation coupled to removal of the $As(GS)_3$ complex from the cytosol by ABC transporters, such as the *Saccharomyces cerevisiae* Ycf1p vacuolar pump (2) or mammalian biliary extrusion pump MRP2 (3). In many mammals, including humans, an alternate metabolic fate of As(III) is methylation in the liver, followed by urinary excretion of the methylated species (4). In the past, this process was considered a detoxification mechanism (5), but more recent data suggest that the methylation actually increases toxicity by producing the more toxic monomethylarsenite [MMA(III)] and dimethylarsenite [DMA(III)], calling into question whether the process is, in fact, a detoxification process (6). An enzyme (termed Cyt19 or As3MT) that catalyzes As(III)-*S*-adenosylmethyltransferase activity has been identified recently in rats and humans (7–9). The enzyme has been characterized *in vitro*, but its physiological role is unknown.

Bacteria and fungi are known to produce volatile and toxic arsines (10) but the physiological roles of arsenic methylation in microorganisms are likewise unclear, and the biochemical basis is unknown. While examining microbial genomes, we identified large number of genes for bacterial and archaeal homologues of Cyt19. We have termed a subset of these genes *arsM* and their protein product ArsM (As(III) *S*-adenosylmethyltransferase). What sets these *arsM* genes apart from genes for other homologues is that they are each downstream of an *arsR* gene, encoding the archetypal arsenic-responsive transcriptional repressor that controls expression of *ars* operons (11), suggesting that these ArsMs evolved to confer arsenic resistance.

The gene for the 283-residue ArsM (29,656 Da) (accession no. NP_948900.1) was cloned from *Rhodopseudomonas palustris* and expressed in an arsenic-hypersensitive strain of *Escherichia coli*. As(III)-resistance cells in *E. coli* expressing recombinant *arsM* correlated with conversion of medium arsenic to the methylated pentavalent species DMA(V) and TMAO and to trimethylarsine [TMA(III)] gas. *In vivo*, the toxicity of inorganic and organic arsenicals is DMA(III),MMA(III) > As(III) > As(V) > DMA(V), MMA(V) > TMAO (12). The major pentavalent products DMA(V) and TMAO are approximately 100-fold and a 1,000-fold, respectively, less toxic than As(III)(13). Although MMA(III) and DMA(III) are more toxic than As(III), they do not accumulate in cells expressing *arsM*. Whereas TMA(III) is more toxic than As(III), its volatility prevents its accumulation in cells expressing *arsM*. The recombinant protein was purified and shown to catalyze transfer of methyl groups from *S*-adenosylmethionine (AdoMet) to As(III), forming di- and trimethylated species. The final product was TMA(III) gas. These results demonstrate that methylation of environmental arsenic by conversion to soluble and gaseous methylated species is a detoxifying process that may contribute to global cycling of arsenic.

Results

Expression of *arsM* Is Transcriptionally Regulated by As(III). To date, 125 bacterial and 16 archaeal ArsM homologues have been identified, most likely AdoMet methyltranferases but of unknown physiological function. The methyltransferases were recently classified into two groups, UbiE/Coq5 S-adenosyl-L-methionine-dependent *C*-methyltransferase and MmtA-like *S*- and *O*-methyltransferases (14). Of these, 12 homologues that belong to the UbiE group (see Fig. 6, which is published as supporting information on the PNAS web site) are adjacent to *arsR* genes, which have been shown to control expression of arsenic-resistance operons (11), implying that their gene products, termed ArsM, play a role in arsenic detoxification. The 12 organisms are diverse, including bacteria and archaea, aerobes and anaerobes, mesophiles, thermophiles, and halophiles. We chose the homologue from the Gram-negative soil bacterium *R. palustris* to characterize the ArsM enzyme *in vitro*. ArsM from *R. palustris* is distantly related to mammalian Cyt19, which exhibits As(III) AdoMet methyltransferase activity *in vitro* (7–9), but whose physiological function is unknown. *R. palustris arsM* is induced by the presence of As(III), antimonite, and As(V) in the medium (data not show). The true inducers recognized by ArsR repressors are the trivalent metalloids, but, *in vivo*, sufficient

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Abbreviations: AdoMet, *S*-adenosylmethionine; As(III), arsenite; As(V), arsenate; DMA(III), dimethylarsenite; DMA(V), dimethylarsenate; GSH, glutathione; ICP, inductively coupled plasma; IPTG, isopropyl β-D-thiogalactoside; MMA(III), monomethylarsenite; NTA, nitriolotriacetic acid; TMAO, trimethylarsine oxide; TMA(III), trimethylarsine.

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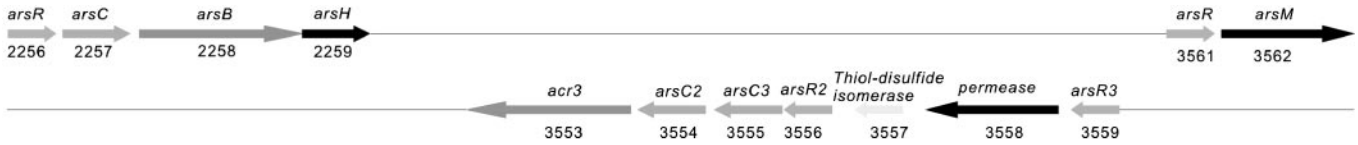

Fig. 1. The *ars* genes of *R. palustris* CGA009. Shown are the operons of *R. palustris* that appear to be regulated by an ArsR-type repressor. The first operon (RPA2256, 2257, 2258, 2259) resembles *ars* operons found in *Pseudomonas* or *Bacillus* of the type *arsRCBH* (ArsR-repressor, ArsC-As(V) reductase, ArsB-As(III) efflux pump, and ArsH-unknown arsenic resistance protein). The *arsRM*-operon (RPA3561, 3562) encodes the As(III)-methyl-transferase (ArsM) described in this report regulated by an ArsR-type repressor. The third operon (RPA3553–RPA3559) of *R. palustris* contains two putative *arsR* genes, two putative As(V) reductases, a putative As(III) permease (*acr3*), and two genes where the function of the proteins is unknown. The two putative As(III) permeases ArsB and Acr3 belong to two unrelated protein families.

As(V) can be reduced to As(III) by ArsC reductases (11), of which there are two in *R. palustris* (Fig. 1).

As(III) Methylation and Volatilization Confers Resistance. Insertional inactivation of *arsM* in *R. palustris* did not result in an increase in As(III) sensitivity compared to the wild type (data not shown), likely due to the presence of multiple arsenic-resistance determinants in *R. palustris*. For that reason, the *R. palustris arsM* gene was cloned and expressed in *E. coli* strain AW3110, which does not have an *arsM* gene. AW3110 is an arsenic-hypersensitive strain of *E. coli* lacking the chromosomal *arsRBC* operon (15). Expression of *arsM* allowed for growth in concentrations of As(III) as high as 0.15 mM after more than a day of incubation (data not shown). ArsM has two nonconserved cysteine residues, Cys-281 and Cys-282, in addition to five conserved cysteine residues (Fig. 6). Because As(III) binds strongly to vicinal cysteine pairs (16), these residues were altered to serine residues, producing the ArsMC2 derivative to improve protein production and future crystallization. The activity and function of ArsMC2 was compared to wild-type ArsM *in vivo* and *in vitro*. Expression of the gene for ArsMC2 conferred resistance similar to that of the wild type (Fig. 2), indicating that this vicinal pair, which is not conserved in homologues, does not play a role in methylation. Growth was often preceded by a lag period that was prolonged in higher concentrations of As(III). The lag was longer than the induction period for ArsM (Fig. 2), suggesting that extended ArsM activity is required before the cells can begin to grow, indicating that As(III) must be metabolized slowly until it decreases to a level permitting growth.

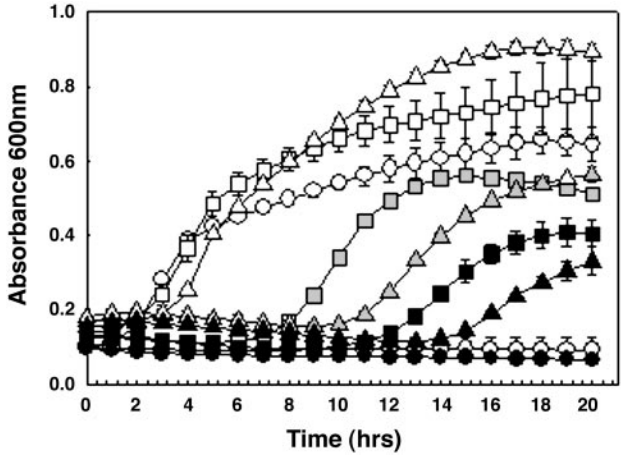

Fig. 2. ArsM confers resistance to As(III) in *E. coli*. Resistance to As(III) was assayed in cells of *E. coli* AW3110(DE3) (ΔarsRBC), bearing vector plasmid pET28a(+) (circle), pET28*arsM* (encoding wild-type ArsM) (square), or pET28*arsMC2* (encoding the ArsMC2 variant) (triangle) were grown with no (white), 50 μM (gray), or 70 μM (black) sodium As(III) at 37°C, with shaking. Absorbance at 600 nm was monitored by using a SPECTRA max 340PC microplate reader (Molecular Devices) with a path length of 0.24 cm. Shown is the average of three independent assays with standard deviation.

When cells were incubated with As(III) for extended periods, the total amount of arsenic in the culture decreased. The reaction was repeated in a closed vial, and gaseous products were trapped on filters saturated with 6% H_20_2 and analyzed for arsenic by HPLC-inductively coupled plasma (ICP)-MS. The amount of volatilized arsenic correlated with the disappearance of solution arsenic (Fig. 3*A*). The only arsenical species found on the filter after oxidation by H_2O_2 was TMAO, which unambiguously identifies the gas as TMA(III). The decrease in As(III) correlated with the appearance of three soluble products in the medium, DMA(V), TMAO, and an unidentified product that did not correspond to any of arsenic standards and TMA(III) in the head space of the reaction vial (Fig. 3*B*). These results are strong evidence that formation of methylated arsenicals is responsible for resistance.

Purified ArsM Is an As(III) AdoMet Methyltransferase. Wild-type ArsM and the C281/282S ArsMC2 variant, both with a His_6 tag, were expressed in *E. coli* and purified in a single step by Ni(II)-nitrilotriacetic acid (NTA) chromatography. As(III) methylation was assayed by HPLC-ICP-MS. In the absence of glutathione (GSH), no methylated species were formed (Fig. 4*A*). Similarly, no methylation was observed in the absence of AdoMet (data not shown). In the presence of ArsM, AdoMet, and GSH, As(III) was converted to DMA(V) (Fig. 4*B*). No MMA(V) was observed, nor did ArsM methylate MMA(III) (data not shown). In contrast, if DMA(III) was used as a substrate, TMAO was formed in a time-dependent manner (Fig. 4*C*), suggesting that ArsM catalyzes formation of trimethylated arsenicals and that the conversion to DMA(V) in Fig. 4*B* might be due to incomplete reaction. When the concentration of wild-type ArsM (Fig. 5 *A* and *C*) or ArsMC2 (Fig. 5 *B* and *D*) was increased ≈10-fold, TMAO was formed in the reaction solution (Fig. 5 *A* and *B*), and an arsenical gas was found in the headspace of the reaction vessel (Fig. 5 *C* and *D*), whether the wild-type ArsM or the ArsMC2 mutant was used. Because H_2O_2 oxidized the gas to TMAO, the gas must have been TMA(III). The amount of TMAO in solution was higher at 3 h than at 17 h, and the amount of TMA(III) in the headspace was higher at 17 h than at 3 h, consistent with rapid methylation of As(III) to DMA(V) [probably through a transient intermediate of MMA(V)] and then slower reduction and methylation of DMA(V) to form TMAO. The pentavalent trimethylated species is then reduced to the final product, gaseous TMA(III), which volatilizes into the headspace of the reaction vial and is trapped on the filter as TMAO by oxidation with H_2O_2. These results are consistent with sequential formation of mono-, di-, and trimethylated arsenicals, with conversion of As(III) to DMA(V) faster than conversion of DMA(V) to TMAO or TMA(III), as proposed by Challenger (17). The data demonstrate that ArsM is a methylase, but it is not clear whether ArsM also catalyzes reduction of DMA(V) to DMA(III) or TMAO to TMA(III), or whether the reduction *in*

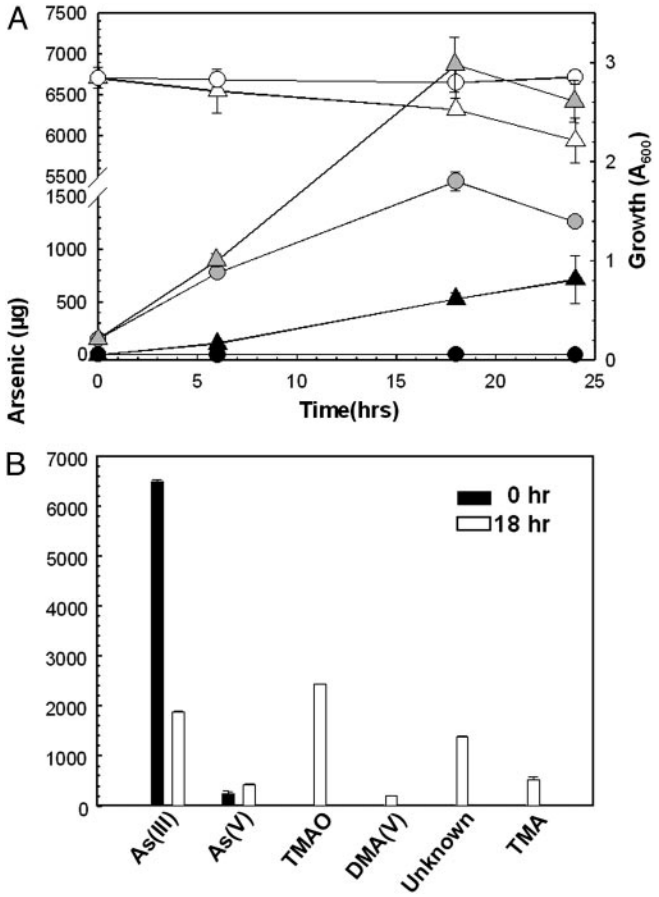

Fig. 3. *In vivo* formation of methylated arsenicals. (*A*) Volatilization of arsenic. Cells of *E. coli* strain AW3110(DE3) with vector plasmid pET28a(+) (circle) or plasmid pET28*arsMC2* (triangle) were grown in 4 ml of LB medium in the presence of 25 μM sodium As(III), and growth was monitored as optical density at 600 nm (gray). Total arsenic in the culture medium (white) and volatilized arsenic (black) were determined as described above. (*B*) Speciation of arsenic in the culture medium. Cultures were grown for 18 h after the determination of the arsenic species in the medium by HPLC-ICP-MS using known standards of As(III), As(V), DMA(V), and TMAO. The unknown peak did not correspond to any of the standards. Black bars indicate the arsenic species at time point 0, white bars, after 18 h of cultivation.

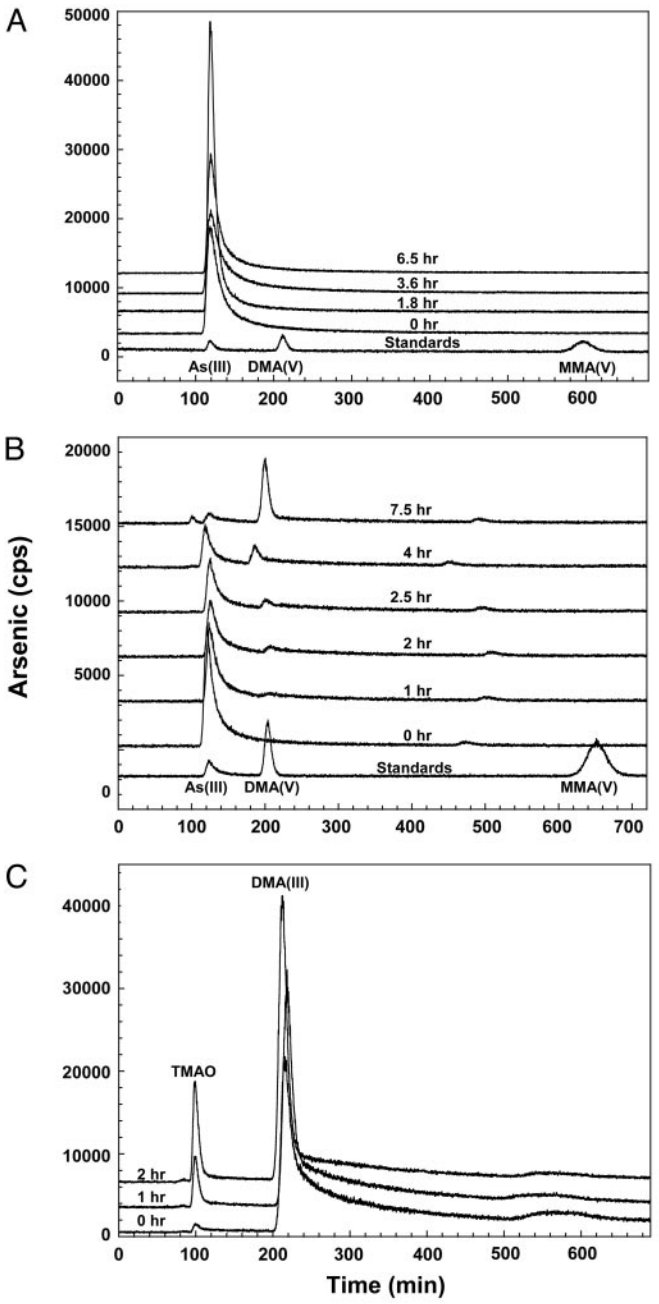

Fig. 4. Purified ArsM catalyzes As(III) methylation. Arsenic speciation of the reaction solution was performed by anion exchange HPLC-ICP-MS, with the relative amounts of arsenic expressed as cps. As indicated, each assay contained 5 μM As(III) or DMA(III), 0.5 mM AdoMet, 8 mM GSH, and 0.8 μM wild-type ArsM in 50 mM phosphate buffer, pH 7.4. Shown are the arsenic species produced with As(III) as substrate without GSH (*A*), As(III) as substrate with GSH (*B*), and DMA(III) as substrate with GSH (C).

vitro is nonenzymatic. GSH will nonenzymatically reduce As(V) to As(III) *in vitro* (18), but reduction *in vivo* requires an enzyme, such as the ArsC As(V) reductase (19). At this stage, it is not apparent why the methylation rates were slow *in vitro*. It is possible that an as yet unidentified methylarsenate reductase works in concert with ArsM *in vivo*.

Discussion

The methylation of As(III) has been observed in a number of organisms, including humans. For example, higher eukaryotes and bacteria have been reported to produce MMA(V) or DMA(V), and fungi produce trimethylarsine (10). Methanogens and aerobic eubacteria have also been suggested to form methylated arsines (20, 21). However, the physiological significance of arsenic methylation is unclear. At first, methylation was thought to be related to arsenic detoxification, because the pentavalent species are relatively innocuous, but, more recently, the process has been suggested to increase arsenic toxicity, because the trivalent species are more toxic than inorganic As(III) (22). Purified mammalian Cyt19 catalyzes arsenic *S*-adenosylmethyltransferase activity (23, 24), but the function of this enzyme *in vivo* is unknown. Did this enzyme evolve for arsenic detoxification or for another unrelated function? Because the rate of As(III) methylation in cultured primary human hepatocytes does not directly reflect variations in basal mRNA levels for Cyt19 (25), this activity may be an adventitious methylation reaction unrelated to mammalian physiology.

In contrast, our data show that As(III) methylation in prokaryotes is a previously undescribed detoxification mechanism. The transcriptional regulation of the *R. palustris* methyltransferase gene shows a direct physiological response to environ-

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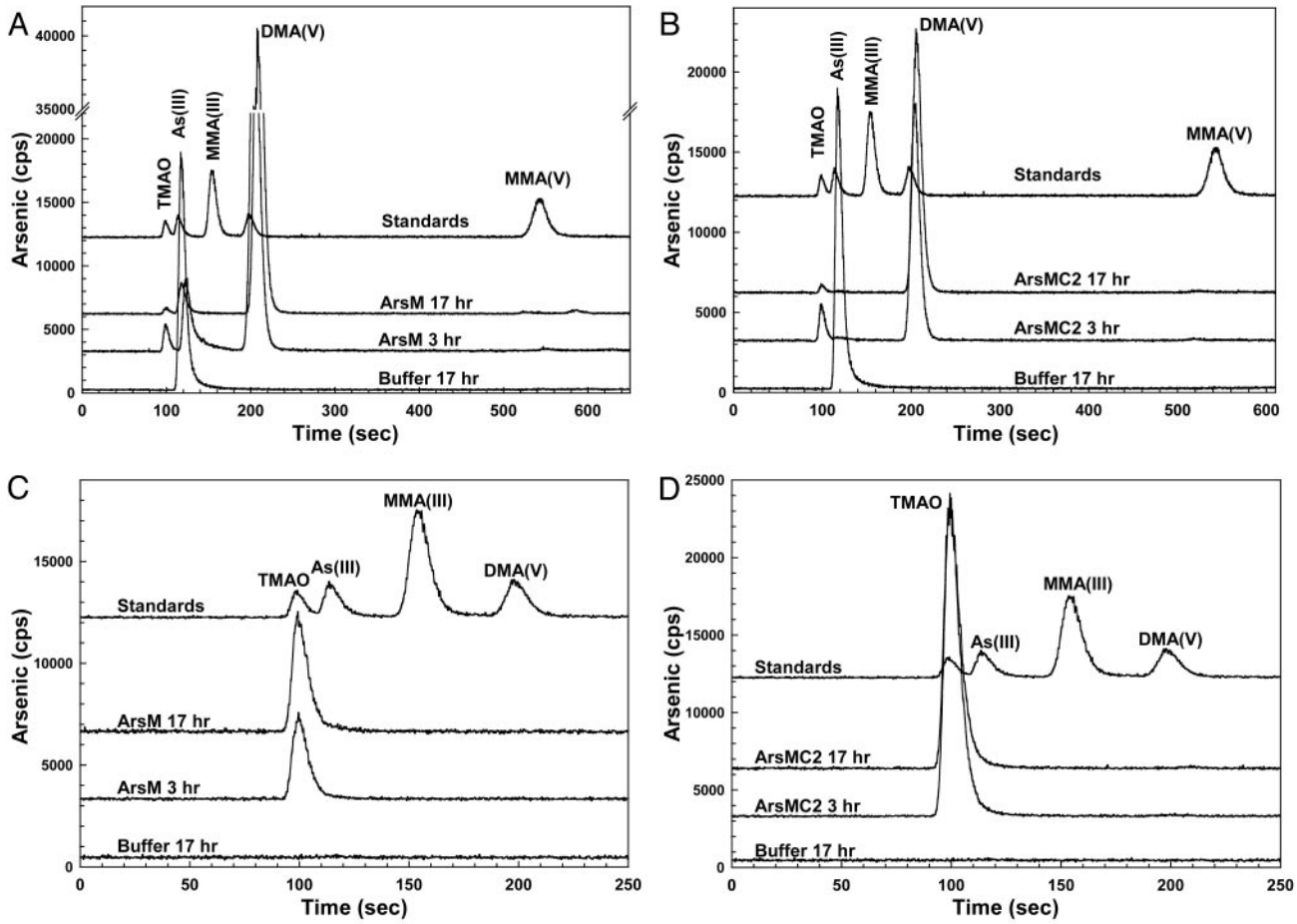

Fig. 5. Formation of volatile TMA(III) by purified ArsM. The activity of wild-type ArsM and mutant ArsMC2 were assayed as described in the legend to Fig. 4, except that the concentration of ArsM was increased to 10 μM. Wild-type ArsM was used in *A* and *C*, and mutant ArsMC2 was used in *B* and *D*. Arsenic speciation of the reaction solution (*A* and *B*) and volatilized gas (*C* and *D*) was performed by anion exchange HPLC-ICP-MS. The reaction products were analyzed after 3 h and 17 h in the presence of ArsM or buffer only.

mental arsenic (data not shown). In response, the cells methylate As(III) to the relatively nontoxic pentavalent species DMA(V) and TMAO. The more toxic MMA(III) and DMA(III) are probably transient intermediates that do not accumulate. Even though the final product is trivalent TMA(III), its volatilization lowers the arsenical concentration in both the medium and cytosol, augmenting detoxification. This arsenical resistance is reminiscent of selenium detoxification, in which volatile dimethyl selenide and dimethyl diselenide are formed in bacteria and plants (14, 26), and of MerA-mediated mercury detoxification, in which Hg(II) is reduced to volatile Hg(0) (27).

Methylation of As(III) by ArsM homologues contributes to the global cycling of arsenic. Bacteria and fungi, which also have ArsM homologues, methylate arsenic to volatile species such as TMA (10). This biogenic source of gaseous arsenic has been estimated to produce 8-fold more atmospheric arsenic than does continental dust (28). TMA(III) is also an intermediate in the formation of other organoarsenicals. *O*-phosphotidyltrimethylarsonium lactic acid, which is found in marine algae and animals, is formed by nucleophilic reaction of phosphoenolpyruvate and TMA(III) (29). A degradation product of *O*-phosphotidyltrimethylarsonium lactic acid is arsenobetaine, which is found in marine animals, such as Western rock lobster and dusky shark, and arsenosugars found in brown kelp could also be derived from TMA(III) (30). Thus, arsenic methylation may be a protective mechanism for individual organisms and is an important link in the global arsenic cycle (31).

Materials and Methods

Chemicals. As(V), As(III), and DMA(V) were obtained from Sigma, MMA(V) was obtained from Chem Service (West Chester, PA), MMA(III) was a gift from Miroslav Styblo (University of North Carolina, Chapel Hill, NC), TMAO was a gift from Tim McDermott (Montana State University, Bozeman, MT). The reagents used for HPLC-ICP-MS, including tetrabutylammonium hydroxide and malonic acid, were obtained from Sigma.

Bacterial Strains, Media, and Growth Conditions. *R. palustris* strain CGA009 was a gift from Caroline Harwood, University of Iowa (Iowa City, IA). Cultures were grown at 30°C anaerobically in the light in photosynthetic medium (32). Sterile solutions of sodium succinate and sodium bicarbonate were added to final concentrations of 10 mM after autoclaving of the medium. Agar was autoclaved separately. When appropriate, kanamycin and tetracycline were added to final concentrations of 0.2 mg/ml and 25 μg/ml, respectively.

E. coli strains were grown aerobically at 37°C in LB medium (33), supplemented with 0.1 mg/ml ampicillin, 25 μg/ml kanamycin, 12.5 μg/ml tetracycline, or 25 μg/ml chloramphenicol, as required. Strain DH5α (Promega) was used for plasmid construction and replication, and strain S17-1 (Biomedal, Seville, Spain) served as the plasmid donor in conjugation with *R. palustris*. Strain AW3110(DE3) (Δ*arsRBC*; ArsR-repressor;

ArsB-As(III) efflux pump; and ArsC-As(V) reductase) (15), which is hypersensitive to As(III), was used for complementation studies. Strain BL21(DE3) (Novagen) was used for protein expression. Bacterial growth was monitored by measuring the optical density at 600 nm.

DNA and RNA Manipulations. Genomic DNA of *R. palustris* was isolated by using Genomic-Tip 20/G and Buffer set (Qiagen, Valencia, CA) following the manufacturer's protocol. Isolation of plasmid DNA was performed by using a QIAPrep Spin Miniprep kit (Qiagen). Total RNA of *R. palustris* was isolated by using an RNeasy kit (Qiagen) following the manufacturer's protocol. Because of the extremely high GC content (65% on average) of the *R. palustris* genome, PCR reactions were modified by supplementation of 10% (vol/vol) DMSO when using *R. palustis* DNA as templates to enhance amplification of DNA targets. Transfer of plasmid vectors into *R. palustris* cells was accomplished by bacterial mating with *E. coli* S17-1 donor cells (34).

Construction of *arsM* Overexpression Vectors. To express ArsM from *R. palustris* in *E. coli*, a 0.85-kb fragment containing the ATG start codon and excluding the stop codon was PCR amplified from *R. palustis* genomic DNA by using the primers 3′ *arsM* (AAACTGCAGCCCGCAGCAGCGCGCCG) (KpnI site underlined) and 5′ *arsM* (AAAGGTACCATGCCCACTGACATGCAAGACGTGA) (PstI site underlined). After digestion with KpnI and PstI, *arsM* was cloned into KpnI/PstI-digested vector pASK-IBA3 (IBA Göttingen, Germany), resulting in plasmid pASK*arsM*. Subsequently, a 0.85-kb NcoI–HindIII fragment containing the *arsM* gene (primer 5′ *arsMw*: GGTGGTCCATGGCCACTGACATGCAAGACGTGAAG (NcoI site underlined) and 3′ *arsMw*: GCGGCCAAGCTTAATGATGATGATGATGATGCCCGCAGCAGCGCGCCGGCACC (HindIII site underlined)) was PCR amplified from pASK*arsM* and inserted into pET28a(+), generating plasmid pET28*arsM*, in which the *arsM* gene is under the control of the *T7* promoter. The primer 5′ *arsMw* introduces the codons for His_6 residues exactly after the last amino acid residue at the C terminus of ArsM, creating a C-terminal His-tagged ArsM of 289 amino acid residues and mass of 30,452 Da. To shorten the fusion, the NcoI site on vector pET28a(+) was used, which changed the second amino acid residue from proline to alanine. A 0.58-kb AscI–HindIII fragment of the *arsM* gene was PCR amplified from pET28*arsM* and substituted with a AscI–HindIII fragment generated by using primer 5′ *arsMC2* (GTCGGCGCGCCGGGTCGGCCCGACCGGCAAAGCCTACGGCCT) (AscI site underlined) and 3′ *arsMC2* (GGTGGTAAGCTTAATGATGATGATGATGCCCGGAGGAGCGCGCCGGCACCTCGCCGTCC) (HindIII site underlined) to produce the *ArsMC2* mutant. This mutant, in which Cys-281 and Cys-282 were altered to serine residues, was constructed to examine whether those two cysteines were required for ArsM activity. Because the properties of ArsMC2 and wild-type ArsM enzymes turned out to be comparable (see below), in some experiments, the derivative was used in place of the wild-type enzyme. All of the sequences were verified by DNA sequencing.

Construction of an *arsM* Gene Deletion in *R. palustris*. The *arsM* gene was deleted by homologous recombination by using the *cre-lox* system (35). Primers 5′ *arsM1* (AAAGAATTCAAGCCGGCTCGCGCGAAGA) (EcoRI site underlined), 3′ *arsM1* (AAACCATGGTGTCAGTGGGCATTGCGGTT) (NcoI site underlined), 5′ *arsM2* (AAAGGGCCCAGACTTCTCAGCCCGTCATTGC) (ApaI site underlined), and 3′ *arsM2* (AAAGAGCTCCAGCAACCAATGACACTCAGGC) (SacI site underlined) were used to amplify 0.6-kb DNA fragments directly up- and downstream of *arsM* by PCR. These two fragments, designated *arsM1* (upstream of *arsM*) and *arsM2* (downstream of *arsM*), were cloned into the plasmid vector pGEM-T EASY (Promega), leading to pGEM*M1* and pGEM*M2* for sequence verification. The *arsM2* gene was subcloned into plasmid pCM184 (35) upstream of the kanamycin cassette at the ApaI/SacI sites, creating plasmid pCM*M2*. Subsequently, *arsM1* was EcoRI/NcoI digested and subcloned into pCM*M2* downstream of the kanamycin cassette, leading to plasmid pCM*M21*. By using *E. coli* S17-1, pCM*M21* was conjugated into *R. palustris* CGA009, and *arsM* was replaced by the kanamycin cassette in a double-recombination event. The deletion was verified by PCR and Southern blotting.

Northern Blot Analysis. A single colony of *R. palustris* was inoculated into 5 ml of photosynthetic medium (PM) and incubated at 30°C for one week. The culture was diluted 1:500 into PM medium supplemented with 10 μM sodium As(III), 0.1 mM sodium As(V), or 5 μM potassium antimonyl tartrate and incubated until the cultures attained an OD_{600} of 0.5, after which higher concentrations of sodium As(III) (0.1 mM), sodium As(V) (1 mM), or potassium antimonyl tartrate (0.05 mM) were added to the respective cultures. After an additional 6 h, total RNA was isolated, separated by formaldehyde gel electrophoresis, transferred to a Hybond-A nylon membrane, and hybridized with 100 μg/ml [γ-^{32}P]-labeled single-stranded *arsM*-DNA probe by using the RadPrime DNA labeling system (Invitrogen) at 42°C overnight.

Resistance Assays. Single colonies of *E. coli* strain AW3110(DE3) (Δ*ars*) and AW3110(DE3) pET28*arsM* were inoculated into 5 ml of LB medium supplemented with the appropriate antibiotics and incubated at 37°C overnight. Late exponential phase cells were diluted 50-fold into 50 ml of LB medium containing 25 μg/ml kanamycin, 0.3 mM isopropyl β-D-thiogalactoside (IPTG), and the indicated concentrations of sodium As(III). Growth was monitored as optical density at 600 nm and continued until the cultures reached stationary phase.

ArsM Purification. Cells of BL21(DE3) pET28*arsM* were grown at 37°C in LB medium to an OD_{600} of 0.5, at which point 0.3 mM IPTG was added to induce expression of His-tagged ArsM or ArsMC2. The cells were grown for another 4 h, harvested by centrifugation (5,000 $\times$ *g*) at 4°C for 20 min, washed once with a buffer A (50 mM Mops, pH 7.5, containing 20% (wt/vol) glycerol, 0.5 M NaCl, 20 mM imidazole, and 10 mM 2-mercaptoethanol), and suspended in 5 ml of buffer A per g of wet cells. The cells were lysed by a single pass through a French-press cell at 20,000 psi, and 2.5 μl per g wet cell of di-isopropyl fluorophosphate was added immediately. Membranes and unbroken cells were removed by centrifugation at 150,000 $\times$ *g* for 1 h, and the supernatant solution was loaded at a flow rate of 0.5 ml/min onto a Ni(II)-NTA column preequilibrated with buffer A. The column was then washed with 150 ml of buffer A, followed by elution with 60 ml of buffer A, with the concentration of imidazole increased to 0.2 M. ArsM was identified by sodium-dodecyl-sulfate polyacrylamide gel electrophoresis. Fractions containing ArsM were concentrated by centrifugation using a 10-kDa-cutoff Amicon Ultrafilter (Millipore). Protein concentrations were estimated by the method of Bradford (Bio-Rad Protein Assay 500-0006) using BSA (Sigma) as a standard.

Arsenic Speciation. Protein was removed from the reaction samples by centrifugation using a 10-kDa-cutoff Amicon Ultrafilter (Millipore). The filtrate was then speciated by HPLC (Series 2000, PerkinElmer) ICP-MS (ELAN 9000, PerkinElmer) using either a reverse-phase C18 column (Jupiter 300) 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 (36) or an anion exchange column (PRP-X100, Hamilton) eluted with a step gradient composed of 9 ml of mobile phase A (20 mM ammonium bicarbonate, pH 8.5) and 18 ml of mobile phase B (20 mM ammonium sulfate, pH 7.0) at a flow rate of 1.5 ml/min.

TMA(III) Analysis. Methylation reactions (4 ml) were performed in capped 20-ml vials with two 2-cm nitrocellulose membrane filters (Schleicher & Schuell) in the cap. The filters were impregnated with 0.15 ml of 6% H_20_2 to oxidize TMA(III) to TMAO. After the reaction, the filters were digested with 0.2 ml of 70% HNO_3 at 70°C for 20 min, which was diluted 20-fold and analyzed by HPLC-ICP-MS.

As(III) Methylation Assays. Methylation of As(III) was assayed both *in vivo* and *in vitro*. *In vivo* assays were performed with *E. coli* strain AW3110(DE3) (Δ*arsRBC*) bearing either vector plasmid pET28a or pET28*arsMC2*. Cultures were grown overnight at 37°C in LB media containing 25 μg/ml kanamycin and 0.3 mM IPTG, and diluted 50-fold into fresh, prewarmed LB media containing 25 μg/ml kanamycin, 0.3 mM IPTG, and 25 μM As(III). The cultures were divided into 4-ml aliquots in capped vials for trapping of TMA(III), as described above, and grown with gentle shaking at 37°C. At the indicated times, the arsenic species in the reaction solution and filters were analyzed in triplicate by HPLC-ICP-MS. *In vitro* assays with purified ArsM were performed in a buffer consisting of 50 mM K_2HPO_2, pH 7.4, containing 8 mM reduced GSH and 0.3 mM AdoMet chloride, unless otherwise indicated.

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