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

Jie Qin*, Barry P. Rosen*, Yang Zhang†, Geijiao Wang†, Sylvia Franke†, and Christopher Rensing†

*Department of Biochemistry and Molecular Biology, Wayne State University, Detroit, MI 48201; and †Department of Soil, Water, and Environmental Science, University of Arizona, Tucson, AZ 85721

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In this article, a mechanism of arsenite [As(III)] resistance through methylation and subsequent volatilization 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 Yct1p vacuolar pump (2) or mammalian biliary excretion 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 monomethylarsenate [MMA(III)] and dimethylarsenate [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-adenosylmethionine methyltransferase 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-adenosylmethionine methyltransferase).

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 methyltransferases but of unknown physiological function. The methyltransferases were recently classified into two groups, UbiE/Coq5 S-adenosyl-L-methionine-dependent C-methyltransferase and MntA-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, nitrolo-triacetic acid; TMAO, trimethylarsine oxide; TMA(III), trimethylarsine.

Present Address: Pacific Northwest National Laboratory, P.O. Box 999, MS P7-50, 790 6th Street, Richland, WA 99352.

Present Address: National Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan, Hubei 430070, China.

To whom correspondence should be addressed at: Department of Soil, Water, and Environmental Science, University of Arizona, Shantz Building #38, Room 429, Tucson, AZ 85721. E-mail: rensingc@ag.arizona.edu.

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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.

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$_2$O$_2$ 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). The only arsenical species found on the filter after oxidation by H$_2$O$_2$ 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(IV), 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. 3B). 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)-nitrotriacetic acid (NTA) chromatography. As(III) methylation was assayed by HPLC-ICP-MS. In the absence of glutathione (GSH), no methylated species were formed (Fig. 4A). 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(IV) (Fig. 4B). 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. 4C), suggesting that ArsM catalyzes formation of trimethylated arsenicals and that the conversion to DMA(IV) in Fig. 4B might be due to incomplete reaction. When the concentration of wild-type ArsM (Fig. 5A and C) or ArsMC2 (Fig. 5B and D) was increased ~10-fold, TMAO was formed in the reaction solution (Fig. 5A and B), and an arsenical gas was found in the headspace. The amount of TMAO in solution was higher at 3 h than at 17 h, and the amount of DMA(IV) in the headspace was higher at 17 h than at 3 h, consistent with rapid methylation of As(III) (Fig. 5A) to DMA(IV) (probably through a transient intermediate of MMA(IV)) and then slower reduction and methylation of DMA(IV) to DMA(IV). The pentavalent trimethylated species is then reduced to the final product, gaseous DMA(III), which volatilizes into the headspace of the reaction vial and is trapped on the filter as TMAO by oxidation with H$_2$O$_2$. These results are consistent with sequential formation of mono-, di-, and trimethylated arsenicals, with conversion of As(III) to DMA(IV) faster than conversion of DMA(IV) to DMA(IV) or TMAO or DMA(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(IV) to DMA(III) or TMAO to DMA(III), or whether the reduction in
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 euobacteria 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 procaryotes is a previously undescribed detoxification mechanism. The transcriptional regulation of the R. palustris methyltransferase gene shows a direct physiological response to environ-
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-phosphotidyltrimethylarsionium 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-phosphotidyltrimethylarsionium 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) (∆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 RNaseasy 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. palustris* genomic DNA by using the primers 3′ *arsM* (AAACTGCAGCCCGCAGCAGCGCCG) (KpnI site underlined) and 5′ *arsM* (AAAGGTACCATGCGCCTGA-CATGCAAGACGTGA) (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 pASKarsM. Subsequently, a 0.85-kb NcoI–HindIII fragment containing the *arsM* gene (primer 5′ *arsM*; GGTGTCCATGCGCCTGA-CATGCAAGACGTGAACCATGCATGCGCCTGAAG (NcoI site underlined) and 3′ *arsM*; CCAGCCGAGAATGCATGCGCCTGAAG (HindIII site underlined)) was PCR amplified from pASKarsM and inserted into pET28a(+), generating plasmid pET28arsM, in which the *arsM* gene is under the control of the T7 promoter. The primer 5′ *arsM* introduces the codons for His6 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 codon from proline to alanine. A 0.58-kb Ascl–HindIII fragment of the *arsM* gene was PCR amplified from pET28arsM and substituted with a Ascl–HindIII fragment generated by using primer 5′ *arsMC2* (GGTGGTAAGCTTAATGA-AACCTGGATC) and 3′ *arsMC2* (GTCGGCGCGC-GCCGGTCGGCCGGCCGGCGCGGCCCCGACGCGCGGCACCGCCTC) (Ascl site underlined) and 3′ *arsMC2* (GGTGGTAAGCTTAATGA-AACCTGGATC) and 3′ *arsMC2* (GTCGGCGCGC-GCCGGTCGGCCGGCCGGCGGCACCGCCTC) (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* (AAAGAATTCAAGGCG-CGCTCGCCGGAAGAGAGTGGCCATGCGCGCGCGCCTC) (EcRII site underlined), 3′ *arsM1* (AAACCATGGTGTCAGTGGGCATTGCGGTT) (NcoI site underlined), 5′ *arsM2* (AAAGGCGAGACCTTCTCAGCCGGCTCATTG) (Apal site underlined), and 3′ *arsM2* (AAAAGGCTCAGGCAACAAATGACACCTCGGAA) (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 pGEMM1 and pGEMM2 for sequence verification. The *arsM2* gene was subcloned into plasmid pCM184 (35) upstream of the kanamycin cassette at the Apal/SacI sites, creating plasmid pCM2M. Subsequently, *arsM* was EcoRI/NcoI digested and subcloned into pCM2M downstream of the kanamycin cassette, leading to plasmid pCM2M1. By using *E. coli* S17-1, pCM2M1 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 antimycin tartrate and incubated until the cultures attained an OD600 of 0.5, after which higher concentrations of sodium As(III) (0.1 mM), sodium As(V) (1 mM), or potassium antimycin 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-32P]-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) (*Δars*) and AW3110(DE3) PET28arsM 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) PET28arsM were grown at 37°C in LB medium to an OD600 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 × 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 × 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 subjected to 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) impregnated with 0.15 ml of 6% H2O2 to oxidize TMA(III) to TMAO. After the reaction, the filters were digested with 0.2 ml of 70% HNO3 at 70°C for 20 min, which was diluted 20-fold and analyzed by HPLC-ICP-MS.

**As(III) Methyltransferase Assays.** Methylation of As(III) was assayed both in vivo and in vitro. In vivo assays were performed with E. coli strain AW3110(DE3) (ΔarsRBC) bearing either vector plasmid pET28a or pET28arsMC2. 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 K2HPO4, pH 7.4, containing 8 mM reduced GSH and 0.3 mM AdoMet, unless otherwise indicated.

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