Biotransformation and Volatilization of Arsenic by Three Photosynthetic Cyanobacteria

Xi-Xiang Yin², Jian Chen², Jie Qin, Guo-Xin Sun, Barry P. Rosen, and Yong-Guan Zhu*

Key Lab of Urban Environment and Health, Institute of Urban Environment (X.-X.Y., Y.-G.Z.), and State Key Lab of Urban and Regional Ecology, Research Center for Eco-Environmental Sciences (X.-X.Y., J.C., G.-X.S., Y.-G.Z.), Chinese Academy of Sciences, Xiamen 361021, China; and Department of Cellular Biology and Pharmacology, Herbert Wertheim College of Medicine, Florida International University, Miami, Florida 33199 (J.C., J.Q., B.P.R.)

Arsenic (As) is a pervasive and ubiquitous environmental toxin that has created worldwide human health problems. However, there are few studies about how organisms detoxify As. Cyanobacteria are capable of both photolithotrophic growth in the dark and heterotrophic growth in the light and are ubiquitous in soils, aquatic systems, and wetlands. In this study, we investigated As biotransformation in three cyanobacterial species (Microcystis sp. PCC7806, Nostoc sp. PCC7120, and Synechocystis sp. PCC6803). Each accumulated large amounts of As, up to 0.39 g kg⁻¹ dry weight, 0.45 g kg⁻¹ dry weight, and 0.38 g kg⁻¹ dry weight when treated with 100 μM sodium arsenite for 14 d, respectively. Inorganic arsenate and arsenite were the predominant species, with arsenate making up >80% of total As; methylated arsenicals were detected following exposure to higher As concentrations. When treated with arsenate for 6 weeks, cells of each cyanobacterium produced volatile arsenicals. The genes encoding the As(III) S-adenosylmethionine methyltransferase (ArsM) were cloned from these three cyanobacteria. When expressed in an As-hypersensitive strain of Escherichia coli, each conferred resistance to arsenite. Two of the ArsM homologs (SsArsM from Synechocystis sp. PCC6803 and NsArsM from Nostoc sp. PCC7120) were purified and were shown to methylate arsenite in vitro with trimethylarsine as the end product. Given that ArsM homologs are widespread in cyanobacteria, we propose that they play an important role in As biogeochemistry.
in the microbiota of such sites (Sinha and Hader, 1996; Ladha and Reddy, 2003; Song et al., 2005). Cyanobacteria are responsible for as much as 50% of earthly photosynthesis and are major contributors to the global oxygen cycle. Metals can exert a potential negative influence on cyanobacterial photosynthesis, such as copper in the atmospheric aerosols (Paytan et al., 2009). Moreover, due to their ability to adapt rapidly to environmental changes and rapid growth rates, cyanobacteria are often key players in toxic algal blooming in various aquatic environments (Bianchi et al., 2000; Dokulil and Teubner, 2000). Nevertheless, there is a dearth of studies on the role of freshwater and soil algae in As biogeochemistry, not to mention the biochemical and molecular mechanisms.

*Synechocystis* sp. PCC6803, *Nostoc* sp. PCC7120, and *Microcystis* sp. PCC7806 are typical freshwater cyanobacteria. All of them are dominant species in the blue algal eruption. However, little is known about As metabolism in these prokaryotic blue-green algae. In this study, we characterized the patterns and molecular mechanisms of As biotransformation in these three species. By rapidly methylating and volatilizing As, these widespread cyanobacteria may be major contributors to the global As cycle.

**RESULTS**

**Arsenic Speciation in Cyanobacteria**

After 2 weeks of exposure to the indicated concentrations of As(III), the resulting As species were analyzed by HPLC-inductively coupled plasma (ICP)-mass spectrometry (MS; Fig. 1). Only As(III) and As(V) were detected after exposure to the lower As(III) concentrations (10 μM), and As(V) was the predominant intracellular species, accounting for 81% to 84% of the total. When exposed to the higher As(III) concentrations (100 μM), both inorganic arsenicals were found as the dominant species. In addition, about 2% to 5% of the total As was in the form of DMAAs(V), demonstrating that this organism is capable of As methylation (Fig. 1). Meanwhile, the As species of the control [free As(III)] were determined.

**Volatile As from Cyanobacteria**

When the three cyanobacteria were treated with either 0.1 or 0.4 mM As(V) for 6 weeks, 11.0 and 22.1 ng (Microcystis), 16.9 and 37.8 ng (Nostoc), and 16.4 and 35.3 ng (Synechocystis), respectively, of volatile arsines, presumably TMAs(III), were trapped. It was noted that the amount of volatile As increased along with increasing external As concentrations, accounting for <0.1% of total As in the medium. At an As(V) concentration of 0.4 mM, production of gaseous As increased sharply. In comparison, the amount of As in the control chemotrap (including the glass tubes, glass wools, and silica gel) was determined (Fig. 2). This demonstrates that the cells have the ability to methylate inorganic As to TMAs(III), the volatile product of the methylation pathway. It is also noted that the production of volatile As was up to the maximum generated by *Nostoc* sp. PCC7120 among three cyanobacteria.

**Cloning and Transcriptional Analysis of the *arsM* Genes**

Using the ArsM sequences from the photosynthetic prokaryote *Rhodopseudomonas palustris* and the eukaryotic photosynthetic alga *Cyanidioschyzon*, we identified a homolog in each of the three cyanobacteria (Fig. 3A). Each gene was 969 bp in length and was designated as *NsarsM* (GenBank accession number HQ891147), *MsarsM* (GenBank accession number HQ891148), and *SsarsM* (GenBank accession number HM776638), respectively. Each was cloned from total genomic DNA from the respective organisms by PCR. Each gene encodes an ArsM homolog of 323 residues, with a predicted molecular mass of 35.26, 35.26, and 35.27 kD. The three ArsM are closely related to RpArsM (283 residues, 29.66 kD; 23.53%, 26.18%, and 25.29%, respectively) and CsArsM (400 residues, 29.66 kD; 23.53%, 26.18%, and 25.29%, respectively). The sequence similarities were higher in the putative S-adenosyl Met binding sites.

To investigate whether exposure to different As concentrations affected the expression level of cyanobacterial *arsM* genes, semiquantitative reverse transcription (RT)-PCR analyses were carried out in the presence and absence of 0.1 mM sodium As(III) (Fig. 3B). Total RNA was extracted from three cyanobacteria. The housekeeping gene *rnpB* was included in all RT-PCR analyses to ensure that equivalent amounts of total RNA were used in all reactions. In the absence of added As (III), none of the *arsM* genes were expressed. In contrast, each was expressed in the presence of 0.1 mM As (III), suggesting that transcription of cyanobacteria.

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**Figure 1.** Arsenic speciation in cyanobacteria after exposure to the indicated concentrations of As(III) for 2 weeks, and the products were analyzed as described in “Materials and Methods”: black bars, As(III); white bars, As(V); gray bars, DMAAs(V). All data are the mean ± SD (n = 3). DW, Dry weight.
arsM genes is regulated by external As(III). This is consistent with the observation that in other bacteria arsM genes are controlled by ArsR As(III) transcriptional repressors (Qin et al., 2006), though the regulation of arsM expression in cyanobacteria needs further investigation.

ArsM Enzymes Detoxify As(III)

To examine whether ArsM enhances As resistance, the arsM genes were cloned into an Escherichia coli vector pET28a and expressed in E. coli strain AW3110, which has no orthologous arsM gene and is hypersensitive to As(III) resulting from the deletion of chromosomal arsRBC operon (Carlin et al., 1995). When grown on As(III)-free Luria-Bertani (LB) medium, no difference could be observed between cells bearing empty vector and expressing ArsM. However, when exposed to 100 μM As(III), cells expressing ArsM grew dramatically better than those bearing empty vector (Fig. 4A). In addition, cells bearing vector plasmid pET28a were sensitive to 50 μM As(III). In contrast, cells expressing the arsM genes could grow in As(III) concentration as high as 100 μM (Fig. 4B), demonstrating that the gene products confer tolerance to As(III).

As(III) Methylation and Volatilization of ArsM

Two different strains of E. coli, BL21(DE3), an As-tolerant strain with a chromosomal ars operon, and AW3110(DE3), an As-hypersensitive strain in which the ars operon was deleted, expressing cyanobacterial arsM biomethylated As(III) primarily to methylated As [DMAs(V) and some amount of TMAsO(V)] after 12 h of incubation by analyzing the soluble As(III) transformation products in the reaction solution (Fig. 5, A and B). It should be noted that expression of arsM in E. coli strain BL21 resulted in more volatilization than expression in AW3110. In addition, the volatile product TMAs(III) was trapped by oxidation.
to TMAsO(V) when expressing arsM genes in the two cell strains (Fig. 5, C and D). Consistent with direct trapping of the gas, more TMAsO(V) was generated by oxidation when arsM was expressed in strain BL21 than in AW3110. In particular, NsArsM showed the highest methylation ability regardless of in which strain the gene was expressed. In addition, the product of the methylation reaction was quantified in cells of the three cyanobacterial strains (Fig. 5, B and C). Moreover, traces of TMAO(V) (< 1%) were detected with expression of NsarsM. These results demonstrate that heterologous expression of the cyanobacterial arsM genes in E. coli conferred the ability to methylate As.

**Purification of ArsM and in Vitro Methylation**

To elucidate the mechanism of As methylation by the algal enzymes, NsArsM and SsArsM were purified from E. coli BL21. Of the three, NsArsM exhibited the highest As(III) methylation activity in vitro. Purified NsArsM methylated As(III) to a variety of species (Fig. 6B). Little As(III) remained, and the primary product was DMAs(V), with lesser amounts of MAs(V). Purified SsArsM produced some MAs(III) (Fig. 6B). Since MAs(III) is the precursor of DMAs(V), this indicates that the reaction rate of SsArsM is slower than NsArsM. TMAs(III) gas could be detected on H₂O₂-impregnated filters by two purified proteins, which oxidize it to TMAsO(V) (Fig. 6C). Less than 2% of the total As was converted to TMAsO(V). These results demonstrate that the two ArsMs exhibit difference in methylation activity in vitro, as was observed in vivo results. Additionally, with both NsArsM and SsArsM, the major product was DMAs(V), indicating that addition of a third methyl group is most likely the rate-limiting step in the overall reaction.

Methylation was determined with two different protein concentrations or two different incubation times using purified NsArsM (Fig. 7). It is noted that more DMAs(V) was produced at the higher concentration of NsArsM or the longer reaction time. In addition, small quantities of TMAsO(V) were detected after 12 h (Fig. 7, B and C).

**DISCUSSION**

Our results clearly demonstrate that three As-tolerant cyanobacteria not only grow at high concentrations of the toxic metalloid but also have the ability to accumulate large amounts of As (Fig. 1). These are possibly related to the organisms’ adaption to their environment. Both prokaryotes, such as *R. palustris*, and eukaryotic algae, such as *Cyanidioschyzon*, are widely distributed in aquatic environments and soils (Qin et al., 2006, 2009), where soluble inorganic As is ubiquitous. As a consequence, these photosynthetic microorganisms have evolved mechanisms to detoxify environmental As. Here, we showed that As(III) is efficiently detoxified by methylation by these three cyanobacteria. Some algae, such as *Cyanidioschyzon, Chlorella vulgaris*, and *Chlamydomonas reinhardtii* have also been shown to accumulate or be resistant to high levels of As (Kaise et al., 1999; Suhendrayatna et al., 1999; Qin et al., 2009). Given the high abundance and ubiquity of cyanobacteria, our results imply that cyanobacteria may contribute substantially to the biogeochemistry of As, particularly under the situation of toxic algal blooming in lakes and ponds also heavily contaminated with As, such as Lake Yangzonghai in the southwest of China.

In microorganisms and plants, As(III) is detoxified by oxidation to relatively less toxic As(V) or formation of organoarsenicals, including methylated arsenicals, arsensugars, and arsenolipids, and volatile As species (Bentley and Chasteen, 2002; Murray et al., 2003). Our results indicate that several of these parallel pathways of As(III) detoxification exist in the three cyanobacteria. First, when exposed to As(III), the cells accumulate primarily As(V) by a process of As(III) oxidation. We
speculated that this As(V) may be located in the vacuole. This is similar to the response of the red alga *Cyanidioschyzon merolae*, which oxidizes As(III) extracellularly, takes up the resulting As(V), and then rereduces it to As(III) for efflux or methylation (Qin et al., 2009). Second, when exposed to high concentrations of As up to 0.1 mM, the relatively nontoxic methylated species DMAs(V) was formed as the major species by ArsM methylation, a parallel pathway that was first identified in *R. palustris* and *Cyanidioschyzon* (Qin et al., 2006, 2009).

Biological production of volatile As compounds is considered to be an important part in the global As biogeochemical cycling (Michalke and Hensel, 2004). Bacteria, fungi, and algae can convert inorganic As into volatile compounds for As detoxification (Qin et al., 2006, 2009; Urík et al., 2007). In this study, the amount of volatile arsenicals depended on the initial As concentrations in the medium (Fig. 2). This may explain why gaseous TMAs(III), the final detoxification product of the ArsM reaction, is produced only when these cyanobacteria are exposed to high concentrations of As.

Figure 5. In vivo formation of methylated arsenicals. A, Speciation of As in the culture medium by cyanobacterial *arsM* genes expressed in *E. coli* BL21. B, Speciation of As in the culture medium of AW3110 expressing cyanobacterial *arsM* genes. C, Volatilization of As from cultures of *E. coli* BL21 expressing cyanobacterial *arsM* genes. D, Volatilization of As from cells of *E. coli* AW3110 expressing cyanobacterial *arsM* genes. Curve 1, Standard; curve 2, Microcystis *arsM*; curve 3, Nostoc *arsM*; curve 4, *Synechocystis arsM*. Cultures were incubated for 12 h in LB medium with 25 μM As(III). E, Speciation of As in the culture medium. Cultures were grown for 12 h. Soluble As species were determined by HPLC-ICP-MS using an anion-exchange column. cps, Counts per second.
To examine whether the ArsM pathway exists in these cyanobacteria, genes encoding three ArsM homologs were cloned and expressed in *E. coli*, where they each conferred As(III) resistance and methylated As(III) to DMAs(V). Two, NsArsM and SsArsM, were purified and shown to catalyze methylation of As(III) to a variety of species in vitro, primarily DMAs(V) and lesser amounts of MAs(III), MAs(V), and TMAs(III) gas, demonstrating that they are genuine ArsM orthologs. To date, only two microbial ArsM homologs, RpArsM from the photosynthetic bacterium *R. palustris* and CmArsM from the eukaryotic alga *C. merolae*, have been characterized. For these organisms, meth-

**Figure 6.** Biomethylation of As(III) by purified ArsM enzymes. A, Purification of ArsM was tested by SDS-PAGE. B, Soluble As species were determined by HPLC-ICP-MS analysis using an anion-exchange column after 12 h of incubation with purified enzyme, as described in “Materials and Methods.” Curve 1, Standard arsenicals; curve 2, starting material; curve 3, 5 μM SsArsM after 12 h at 37°C; curve 4, 5 μM NsArsM after 12 h at 37°C. C, Volatile As species trapped on H₂O₂-impregnated filters and determined by HPLC-ICP-MS analysis using a C18 reverse phase column, as described in “Materials and Methods.” Curve 1, LB medium after 12 h at 37°C without any ArsM; curve 2, 5 μM SsArsM after 12 h at 37°C; curve 3, 5 μM NsArsM after 12 h at 37°C.

**Figure 7.** Identification of methylated As by purified NsArsM. A, Soluble As species were analyzed after 4 h of incubation with purified NsArsM. Curve 1, Starting material; curve 2, 1 μM NsArsM after 4 h at 37°C; curve 3, 2 μM NsArsM after 4 h at 37°C. B, As species were determined after different incubation time with purified NsArsM. Curve 1, Starting material; curve 2, 5 μM NsArsM after 4 h at 37°C; curve 3, 5 μM NsArsM after 12 h at 37°C. C, The products of methylation by 5 μM NsArsM after 4 or 12 h were examined. cps, Counts per second.
ylation of As(III) by ArsM was demonstrated to be a detoxification mechanism, and we have proposed that methylation and volatilization by photosynthetic microbes are significant contributors to global biogeochemical cycling of As (Mukhopadhyay et al., 2002). Moreover, the fact that both in vivo and in vitro results showed that the ArsM from Nostoc exhibits the highest methylation capacity implies that there is opportunity in improving methylation in cyanobacteria by genetic engineering.

In conclusion, based on our data, we speculate that the abilities of three cyanobacteria to accumulate and methylate As could be useful for phytoremediation and phytotransformation of As-contaminated water or soil. Advantages of using these environmentally friendly cyanobacteria include rapid growth plus high levels of accumulation and biotransformation of As to less toxic forms. In addition, given the high abundance of cyanobacteria in freshwater environment and soils, these photosynthetic prokaryotic microbes may play a significant role in the biogeochemical cycling of As in aquatic systems.

MATERIALS AND METHODS

Organism and Cultivation

*Microcystis* sp. PCC7806, *Nostoc* sp. PCC7120, and *Synechocystis* sp. PCC6803 were obtained from the Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China, and were grown in BG-11 medium (Rippka et al., 1979). Experiments were conducted in capped 20-mL vials, and TMAs(III) was trapped by H2O2 oxidation as described above.

Cloning of the *arsM* Gene and Expression in *Escherichia coli*

DNA was extracted from the cultures of each cyanobacterium using a Genomic-Tip 20/G and buffer set following the company’s protocol (Qiagen). Plasmid DNA was isolated with a QIA Prep Spin Miniprep kit (Qiagen). For construction of plasmids for *arsM* gene expression in *E. coli*, three 0.96-kb fragments containing the AFG start codon and the TGA stop codon were amplified from genomic DNA using three pairs of primers: forward primer 5′-CCGCAATGCGTTAACTCCTGAACTAC-3′ (NdeI site underlined) and reverse primer 5′-CCGCTCGGATTAAAGAAAGGCTAC-3′ (XhoI site underlined) for *MarsM†; forward primer 5′-CCGCAATGCGTTAACTCCTGAACTAC-3′ and reverse primer 5′-CCGCTCGGATTAAAGAAAGGCTAC-3′ for *NsarsM†; and forward primer 5′-GGGATCCCATGCTAGTCG-3′ (NdeI site underlined) and reverse primer 5′-CCGCTCGGATTAAAGAAAGGCTAC-3′ (XhoI site underlined) for *SsarsM†. The PCR product was cloned into PMD19T simple vector (Takara Bio) to create the plasmid PMD19T-arsM. After digestion with NdeI and XhoI, the 0.96-kb fragments were inserted into pET28a (+) to create plasmids of pET28a-arsM, in which the *arsM† genes were controlled by the T7 promoter. As a consequence, the N-terminal His tagged ArsMs were established composed of 327 amino acid residues. All of the sequences were verified by DNA sequencing.

Arsenic Speciation in Cyanobacteria

Arsenic was supplied as NaAsO2 at the indicated concentrations (10 and 100 μM) to investigate As transformation in cyanobacteria. Three replicates were done in each set. After exposure for up to 14 d, the cultures were harvested and rinsed with deionized water and ice-cold phosphate buffer [1 μM K2HPO4, 0.5 μM Ca(NO3)2, and 5 mM MES, pH 6.0]. Methylation of As(Ill) was assayed both in vivo in cultures of cyanobacteria. Approximately 0.5 μM arsenic was added as NaAsO2 to each broth. After exposure for 4 h, the cultures were harvested by centrifugation, washed three times with deionized water, and filtered to remove cell debris. The samples were heated in a microwave digestion system (CEM Microwave Technology) and As speciation in extracts was determined by HPLC-ICP-MS (7500a; Agilent Technologies; Zhu et al., 2008). Chromatographic columns were obtained from Hamilton and consisted of a precolumn (11.2 mm; 12–20 μm) and a XR-100 10-μm anion-exchange column (250 × 4.1 mm). The mobile phase consisted of 10 mM dimethyl ammonium hydrogen phosphate [NH4]2HPO4 and 10 mM ammonium nitrate [NH4NO3] adjusted to pH 6.2 using nitric acid.

TMAs(III) Chemotraping

Volatile TMAs(III) released from cyanobacteria was trapped as reported (Mestrot et al., 2009). The preparation of the trap tube was as follows: silica gel (0.5-mm diameter) was immersed in 5% HNO3 overnight, washed with Millipore water, impregnated with 10% AgNO3 solution (w/v) overnight, and dried in an oven at 70°C. Subsequently, the silica gel was loaded into a 3-mL burette and held in by a small quantity of quartz wool at each end. The tube was covered with aluminum foil to avoid photodecomposition of silver nitrate. A vessel was designed for volatile arsonie trap consisting of a triangular flask (250 mL) and a glass joint with inlet and outlet of gas. The trap tube was connected to the one of the outlet, and a super silent adjustable air pump (ACO-9601; 2-W power) was linked to the inlet to supply oxygen for the growth of cyanobacterium. The trapping period lasted for 6 weeks with 100 mL cyanobacterium culture. Then, 5 mL of 1% (v/v) hot boiling nitric acid was used to elute the collected As in traps. Total As was analyzed by ICP-MS.

Purification of ArsM and in Vitro Methylation Assays

Proteins were expressed in *E. coli* strain BL21(DE3) bearing plasmid pET28a-arsM and purified by Ni2+-NTA chromatography with elution by a buffer consisting of 50 μM MOPS, pH 7.5, containing 20% (w/v) glycerol, 0.5 μM NaCl, 200 mM imidazole, and 10 mM 2-mercaptoethanol. Fractions containing SsArsM were concentrated by centrifugation using a 30-kD cutoff Amicon Ultrafilter (Millipore). ArsMs were identified by SDS-PAGE. Fractions containing purified ArsMs were pooled and concentrated. Purified proteins were stored at −70°C until analysis. Protein concentrations were estimated by the method of Bradford (1976) using bovine serum albumin (Sigma-Aldrich) as a standard.

As(Ill) methylation with purified NsArsM and SsArsM was performed in a buffer consisting of 50 mM K2HPO4, pH 7.4, containing 8 mM GSH, 0.3 mM S-adenosyl-Met chloride, 10 μM As(III), and either 5 μM NaSarsM or SsArsM at 37°C for 12 h (Qin et al., 2006). The samples were filtered through 3-kD cutoff Amicon Ultrafilters (Millipore). The As species were analyzed by HPLC-ICP-MS with either a reverse-phase C18 column (5 μM tetrabutylammonium hydroxide, and 5% methanol, pH 5.6, with a flow rate of 1.0 mL/min (Cui et al., 2004) or with an anion-exchange column (PRP-X100; Hamilton) 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. Methylation reactions (4 mL) were performed in capped 20-mL vials, and TMAs(III) was trapped by H2O2 oxidation as described above.

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