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Identification of Steps in the Pathway of Arsenosugar Biosynthesis

Xi-Mei Xue[†], Jun Ye[†], Georg Raber[‡], Barry P. Rosen[§], Kevin Francesconi[‡], Chan Xiong[‡], Zhe Zhu^{||}, Christopher Rensing[⊥], and Yong-Guan Zhu^{*,†,‡,||}

[†]Key Laboratory of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, Xiamen 361021, China

[‡]Institute of Chemistry, NAWI Graz, University of Graz, Graz 8010, Austria

[§]Department of Cellular Biology and Pharmacology, Herbert Wertheim College of Medicine, Florida International University, Miami, Florida 33199, United States

^{||}Department of Chemical and Environmental Engineering, Faculty of Science and Engineering, University of Nottingham, Ningbo 315100, China

[⊥]College of Resources and Environment, Fujian Agriculture and Forestry University, Fuzhou 350002, China

^{*}State Key Laboratory of Urban and Regional Ecology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China

Abstract

Arsenosugars are arsenic-containing ribosides that play a substantial role in arsenic biogeochemical cycles. Arsenosugars were identified more than 30 years ago, and yet their mechanism of biosynthesis remains unknown. In this study we report identification of the *arsS* gene from the cyanobacterium *Synechocystis* sp. PCC 6803 and show that it is involved in

***Corresponding Author** Phone: +86(0)592 6190997; Fax: +86(0)592 6190977; ygzhu@iue.ac.cn.

Supporting Information

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Supporting methods; reagents and solutions (pS5); confirmation of *arsMarsS* operon (pS5); phylogenetic analyses of SsArsS (pS6); comparative growth curves for WT, disruption strains, and complementary strains (pS6); cloning *arsM* and *arsS* from cyanobacteria (pS7); resistance assays (pS8); nucleotide sequence synthesis of ArsS orthologs from other sequenced organisms (pS8); construction of SsarsS cysteine mutants (pS9); immunological detection of ArsS and ArsM (pS10); the structures of several common arsenic compounds found in nature (Figure S1); bacterial strains and plasmids (Table S1); primer sequences used for PCR amplification in the disruption, complementation, gene expression, site mutant, and *ars* operon identification strategies (Table S2); ICP-MS and chromatographic conditions used for arsenic species analysis (Table S3); confirmation of *arsMarsS* operon (Figure S2); Gene deletion and complementation (Figure S3); phylogram of SsArsS and representative enzymes of the radical SAM superfamily with different functions (Figure S4); the UV/visible spectroscopy of SsArsS shows a shoulder centered at 410 nm for the [4Fe-4S]₂⁺ protein (Figure S5); SDS-PAGE analysis (12%, Coomassie blue-stained) of SsArsM and/or ArsS expression in Rosetta bearing the indicated plasmid (Figure S6); SsArsS or/and SsArsM confers resistance to As(III) in *E. coli* (Figure S7); arsenic concentrations of different *E. coli* Rosetta cells or Rosetta cells expressing *SsarsM* and *SsarsS* (Figure S8); ICP-MS and HR-ESMS measurements of unknown arsenic species in *E. coli* medium (Figure S9); HPLC-ICP-MS (A) and HR-ESMS measurements (B) of unknown arsenic species in *E. coli* cells (Figure S10); arsenic species transformed by different combination of *SsarsS* mutants and *SsarsM* (Figure S11); the information on ArsS orthologs studied in this study (Table S4); and multiple alignment of SsArsS and other five ArsS orthologs (Figure S12) (PDF)

Notes

The authors declare no competing financial interest.

arsenosugar biosynthesis. In the *Synechocystis* sp. PCC 6803 *ars* operon, *arsS* is adjacent to the *arsM* gene that encodes an As(III) *S*-adenosylmethionine (SAM) methyltransferase. The gene product, ArsS, contains a characteristic CX₃CX₂C motif which is typical for the radical SAM superfamily. The function of ArsS was identified from a combination of *arsS* disruption in *Synechocystis* sp. PCC 6803 and heterologous expression of *arsM* and *arsS* in *Escherichia coli*. Both genes are necessary, indicating a multistep pathway of arsenosugar biosynthesis. In addition, we demonstrate that ArsS orthologs from three other freshwater cyanobacteria and one picocyanobacterium are involved in arsenosugar biosynthesis in those microbes. This study represents the identification of the first two steps in the pathway of arsenosugar biosynthesis. Our discovery expands the catalytic repertoire of the diverse radical SAM enzyme superfamily and provides a basis for studying the biogeochemistry of complex organoarsenicals.

Graphical Abstract

INTRODUCTION

Arsenoribosides, commonly known as arsenosugars, were first isolated from the brown kelp, *Ecklonia radiata*, and chemically identified in 1981.¹ More than 20 arsenosugars consisting of dimethylated arsenosugars, dimethylated thio-arsenosugars, and trimethylated arsenosugars are known to occur primarily in marine organisms but also in some terrestrial organisms (Figure S1 of the Supporting Information, SI).^{2,3} They are natural constituents of marine organisms and not toxic to marine plants or animals. Thus, the synthesis of arsenosugars represents a mechanism of arsenic detoxification. Arsenosugars also appear to play a central role in the pathway of arsenic biotransformation in marine systems. First, they are likely intermediates in the biosynthesis of the osmolyte arsenobetaine, which is the dominant arsenical in marine organisms and arsenobetaine.^{1,4} The enzymes for glycine betaine synthesis (GbsB/GbsA) have been shown to be involved in the biosynthesis of arsenobetaine from arsenocholine.⁵ Second, arsenosugars are also the likely precursors of arsenosugar phospholipids,^{6,7} which may be components of the algal cell membrane.⁸ Incorporation of arsenolipids increases under low phosphate conditions and may represent a phosphate-sparing mechanism under those conditions.⁹

Dimethylated arsenosugars consist of a dimethylarsinoyl moiety, in which pentavalent arsenic is bound to one oxygen, to two methyl groups and to a 5'-deoxyribose (Figure S1). The dimethylated arsenosugars differ from one another only in structure of the side chains at the 5'-deoxyribose C1 position. A proposed pathway for arsenic transformation in the

marine environment suggests that SAM is the source of is nontoxic in humans.¹ Edmonds and Francesconi proposed that arsenosugars can be converted to arsenocholine by the cleavage of the C₃–C₄ bond of the sugar residue and methylation of the arsenic atom. Further oxidation at the C-4 of the sugar residue results in the production of both the methyl groups and the 5'-deoxyribose group of arsenosugars.⁴

In this study, the *arsS* (hereafter referred to as *SsarsS*) and *arsM* (hereafter referred to as *SsarsM*) genes from *Synechocystis* were disrupted to examine their function in arsenosugar biosynthesis. Either disruption mutant was unable to synthesize arsenosugars, indicating that both genes are involved in the biosynthetic pathway. The *SsarsM* and *SsarsS* genes were heterologously expressed in *E. coli*. Cells expressing *SsarsM* could synthesize dimethylarsenate but not arsenosugar, while cells expressing both genes produced dimethylarsenor-iboside derivatives. Our results elucidate the first two steps in the pathway of arsenosugar biosynthesis. The first step is addition of methyl groups from SAM to As(III) by the SAM methyltransferase SsArsM. The second step is addition of the deoxyribose moiety by the radical SAM enzyme SsArsS. This study demonstrates the first two steps in the pathway of arsenosugars and puts the synthesis of complex organoarsenical on a firm biochemical basis.

MATERIALS AND METHODS

Strains and Plasmids.

Unless otherwise indicated, *E. coli* was cultured aerobically in Luria–Bertani (LB) medium at 37 °C containing 100 µg mL⁻¹ ampicillin, 50 µg mL⁻¹ kanamycin, or 30 µg mL⁻¹ chloramphenicol, as required. *E. coli* AW3110 (DE3) (*arsRBC*)¹⁰ was used for resistance assays. Plasmids pET-28a-*SsarsS*, pET-22b-*SsarsS*, pETDuet-*SsarsS* or/and pET-28a-*SsarsM* were transformed into *E. coli* BL21 (DE3) or Rosetta (DE3) for expression of SsArsM and SsArsS and for production of arsenical products. All strains and plasmids are shown in Table S1.

Disruption of *SsarsS* and *SsarsM* in *Synechocystis* sp. PCC 6803.

A mutant of *Synechocystis* with a disruption of the *SsarsM* gene has been described.¹¹ Mutagenesis of *SsarsS* (*Synechocystis arsS*) was performed (Figure S3A) as follows: (a) *SsarsS* was amplified with primers SsarsSF and SsarsSR (Table S2); (b) the purified *SsarsS* fragment after PCR was cloned into pMD19T simple vector (TaKaRa, Dalian, China) to yield plasmid pMD-*arsS*; (c) a kanamycin resistance (*KanR*) cassette cut from pKW1188¹² with *EcoRI* was inserted into the *EcoRV* site of pMD-*arsS* to create gene-disruption *KanR*-insertion cassette, pMD*arsSkan*; (d) the plasmid was trans-formed into *Synechocystis* WT according to a previous description;¹³ (e) *Synechocystis arsS* was obtained after three serial isolation of single colonies on BG11 agar plates containing 30 µg mL⁻¹ kanamycin; and (f) PCR with diagnostic primers (Table S2) was used to confirm that the *SsarsS* gene was completely replaced. *Synechocystis arsS* was cultivated in 100 mL of BG11 medium supplemented with 50 µg mL⁻¹ kanamycin at 30 °C.

Complementation of *SsarsS* in *Synechocystis* sp. PCC 6803.

Complementation of the *Synechocystis arsS* mutant (*Synechocystis arsS::arsS*) (Figure S3A) was achieved by “knock-in” of intact *SsarsS* containing the *rbcL2A* promoter with a chloramphenicol resistance (*CamR*) gene. An intact *SsarsS* was amplified using primers *arsSc-F* and *arsSc-R* (Table S2). The *rbcL2A* promoter, P_{rbcL2A} , was produced with primers $F\overline{P}CO2noEcoRI$ and $R\overline{P}BBRBS$.¹⁴ The above PCR products were cloned into pMD19T simple vector to produce plasmid p*MarsSc* and p*Mpromoter* respectively, sequenced to confirm the integrity of the cloned flanking DNA. The plasmid containing *SsarsS* with the promoter was constructed by inserting P_{rbcL2A} into the *EcoRI* and *SpeI* sites of the plasmid p*MarsSc*. A *CamR* gene amplified from pACYCDuet-1 (Novagen, Madison, WI) using primers *cmr-F* and *cmr-R* was digested with *NheI* and *HindIII*, cloned into the upstream of P_{rbcL2A} . Finally, the fragment consisting of a *CamR* gene, P_{rbcL2A} , and *SsarsS* was linked with a plasmid pKW1188 without *KanR* by T4 DNA ligase (MBI Fermentas, Flam-borough, ON, Canada) to prepare plasmid p*KcmrparsS*. This plasmid was used to transform *Synechocystis arsS*, and the above fragment was inserted into the neutral site *slr0168*. In all cases, *Synechocystis arsS::arsS* was selected on BG11 agar plates containing 30 $\mu\text{g mL}^{-1}$ kanamycin and 15 $\mu\text{g mL}^{-1}$ chloramphenicol. PCR amplification with the genomic DNA from *Synechocystis arsS::arsS* as templates and the primers *gp267-9* and *gp267-10*¹⁵ was carried out to confirm that an intact *SsarsS* was present in *Synechocystis arsS::arsS* (Figure S3B). The resulting *Synechocystis arsS::arsS* strain was cultivated in 100 mL of BG11 medium with 50 $\mu\text{g mL}^{-1}$ kanamycin and 30 $\mu\text{g mL}^{-1}$ chloramphenicol.

As(III) Transformation Assays.

After one or 2 weeks of continuous illumination culture, 50 or 100 mL of cyanobacteria (*Synechocystis* WT, *Synechocystis arsS*, and *Synechocystis arsS::arsS*) were collected by centrifuging and washing twice with cold 2-morpholinoethane sulfonic acid buffer.¹⁶ About 20 mg of lyophilized cells were added into 1.5 mL Eppendorf tubes with 1 mL of a mixture of $\text{CH}_3\text{OH}(\text{MeOH})/\text{H}_2\text{O}$ (1 + 1, v/v). The cells were extracted by placing the tubes in an ultrasonic bath for 20 min followed by rotating on a rotary wheel overnight. The extracts were centrifuged for 10 min at 13 520g. The supernatant was purged with N_2 to remove MeOH, and then filtered through a 0.22 μm membrane filter into 1 mL crimp/snap polypropylene vials (Agilent Technologies, Palo Alto, CA, U.S.A.) for arsenic species analysis.

The transformation of As(III) under the control of *ArsS* or/ and *ArsM* was assayed in vivo. The assays were performed with Rosetta bearing pET-28a-*SsarsM*, Rosetta bearing pETDuet-*SsarsS*, Rosetta bearing empty pETDuet-1 and pET-28a, or Rosetta bearing pET-28a-*SsarsM* and pETDuet-*SsarsS*. Cells were grown overnight at 37 °C with shaking (200 rpm) in LB medium containing corresponding antibiotic, diluted 100-fold into 5 mL of fresh LB medium. After incubation at an $\text{OD}_{600\text{ nm}}$ of 0.5 was carried out with 0.5 mM IPTG for 3 h, cells were treated with 1 μM , 10 μM , 100 μM , 1 mM, or 3 μM of either As(III) or As(V). At the indicated times, the cells and culture medium were collected.

Arsenic Species Analysis.

High performance liquid chromatography-inductively coupled plasma mass spectrometry (HPLC-ICP-MS) measurements on arsenic species were performed using an Agilent 7500cx ICP-MS (Agilent Technologies, California, U.S.A.) for element detection coupled with an Agilent 1200 HPLC system. Anion-exchange chromatography separation was performed using PRP-X100 with a guard column (Hamilton, Reno, NV, U.S.A.). A Shiseido CAPCELL PAK C18 MGII (Shiseido, Tokyo) with a matching guard column was employed for ion-pair reversed-phase chromatographic separation. All chromatography parameters are shown in Table S3.

Unknown arsenic compounds were identified by using HPLC-ICP-MS/electrospray ionization tandem mass spectrometry (ESI-MS-MS, Agilent 6460, Agilent Technologies, Waldbronn, Germany), and high-resolution mass spectrometer (HPLC-ESI-HR-MS-MS) which performed in positive mode on a Q-Exactive Hybrid Quadrupole-Orbitrap MS after arsenic species separated on a Dionex Ultimate 3000 series column (Thermo Fischer Scientific, Erlangen, Germany).

RESULTS

arsS Gene Involved in Arsenosugar Biosynthesis.

Our search for candidate genes involved in arsenosugar biosynthesis was guided by the hypothesis that oxidation of DMAs(III) by addition of the deoxyadenosyl radical group from SAM and enzymatic hydrolytic removal of adenine would result in the formation of arsenosugars in marine algae.⁴ *Synechocystis* produces two species of arsenosugars, indicating that this microbe has the biosynthetic enzymes for arsenosugar synthesis.^{11,17} We previously identified the enzyme SsArsM as an As(III) *S*-adenosylmethionine methyltransferase in *Synechocystis*.¹⁶ The *SsarsM* gene is adjacent to two other open reading frames, *slr0304* and *slr0305* (Figure 1). A protein BLAST search using SLR0304, which we termed as SsArsS, as a query revealed the presence of regions conserved in the radical SAM superfamily, in particular iron–sulfur cluster (FeS) and SAM binding sites. The *SsarsM* and *SsarsS* genes comprise an *ars* operon (Figure S2). SLR0305 is related to the family of SNARE-associated Golgi membrane proteins, and this gene is cotranscribed with the other two genes, but it is not clear if it has an arsenic-related function. *Nostoc* sp. PCC 7120, which also produces arsenosugars,¹⁸ has a putative *arsMarsS* operon. The *Nostoc* operon lacks a gene corresponding to SLR0305, indicating that the latter does not have an arsenic-related function. We hypothesize from the linkage of the *arsM* and *arsS* genes in more than one arsenosugar producers that these two genes have related functions encoding a biosynthetic pathway for arsenosugar biosynthesis (Figure 1). We previously demonstrated that a strain in which *SsarsM* was disrupted cannot synthesize arsenosugars, supporting our hypothesis that DMAs production by ArsM is the first step in arsenosugar synthesis.¹¹

We hypothesize that ArsS catalyzes the second step in arsenosugar biosynthesis by transfer of a deoxyadenosyl group from SAM to DMAs. This could be the first committed step in the biosynthetic pathway because the product of ArsM, DMAs can have other biosynthetic fates. ArsS is a member of the radical SAM superfamily. Radical SAM enzymes are characterized

by a CX₃CX₂C motif that provides co-ordinations for binding of a unique four iron-four sulfur ([4Fe-4S]) cluster, although the spacing of the cysteines in this motif can vary.¹⁹ Both the alignment of SsArsS with those of functionally characterized radical SAM enzymes and construction of a molecular phylogenetic tree indicate that SsArsS is closely related to MoaA (Figure S4), which harbors an N-terminal [4Fe-4S] cluster involved in a 5'-deoxyadenosyl radical generation and a C-terminal [4Fe-4S] cluster involved in substrate binding and/or activation in conversion of 5'-GTP to an oxygen-sensitive tetrahydropyranopterin.²⁰ We have used UV/visible spectroscopy to examine a visible absorption band at 410 nm for the [4Fe-4S]²⁺ protein (Figure S5).²¹

SsArsS Involved in the Biosynthesis of Arsenosugars.

To examine the involvement of ArsS in arsenosugar biosynthesis, we disrupted the *SsarsS* gene of *Synechocystis* by inserting a *KanR* cassette inside *SsarsS*. We evaluated the ability of the parental wild type strains and its derivatives to grow. No difference in growth rate in the absence of As(III) was observed among wild type, *Synechocystis arsS* and *Synechocystis arsS::arsS* strains (Figure S3C), indicating that ArsS is not required for viability under these growth conditions. The production of arsenosugars by wild type and mutant strains in medium containing different arsenic species was determined by HPLC-ICP-MS analysis. Consistent with earlier studies,^{11,17} wild-type *Synechocystis* exposed to As(III) produces As(V) as the predominant soluble arsenic species, and small amounts of MAs(V), DMAs(V), and arsenosugars (Figure 2a). Again, it is not possible to deduce whether the products of the reaction were trivalent species that oxidized in air to their pentavalent forms. In addition, wild type *Synechocystis* transforms MAs(V) or DMAs(V) into arsenosugars (Figure 2c, e). *Synechocystis arsS* was unable to generate arsenosugars in medium containing either As(III) or pentavalent methylated arsenic species (Figure 2b, d, f). The *Synechocystis arsS::arsS* strain regained the ability to produce arsenosugars (Figure S3D). These results clearly showed that SsArsS is likely involved in arsenosugar biosynthesis.

Heterologous Expression of SsarsS and SsarsM.

To demonstrate the linked function of *SsarsM* and *SsarsS* in arsenosugar production, the *SsarsS* and *SsarsM* genes were heterologously expressed alone or in combination in *E. coli* Rosetta (DE3), which has neither in its genome (Figure 3A and Figure S6A). Heterologous expression of *arsM* genes in *E. coli* strain AW3110 (DE3), in which the chromosomal *ars* operon was deleted¹⁰ has been shown to confer As(III) resistance.^{22,23} Similarly, expression of *SsarsM* alone in *E. coli* strain AW3110 (DE3) conferred resistance to As(III) (Figure S7). Surprisingly, expression of *SsarsS* alone conferred As(III) resistance (Figure S7). SsArsS has 11 cysteine residues that might bind sufficient As(III) to confer moderate resistance or some free radicals generated by the process of As(III) oxidation to As(V) by SsArsS when the gene is expressed at high levels.

ICP-MS was also used to quantify total arsenic in *E. coli* Rosetta cells (Figure S8A) treated with 0.5 mM IPTG and 1 μ M As(III) for 20 h. Rosetta cells expressing *SsarsS* accumulated the most arsenic, up to 83 mg kg⁻¹. Cells expressing *SsarsM* alone accumulated less (44 mg As kg⁻¹), which was similar to cells expressing both genes (39 mg As kg⁻¹). The arsenic content of cells expressing both *SsarsM* and *SsarsS* peaked after exposure for 3 h, and

subsequently declined, suggesting that they expelled their arsenic compounds from the cells (Figure S8B).

The arsenic species in medium were analyzed by HPLC-ICP-MS after *E. coli* Rosetta cells exposed to 3 μ M As(III) for 12 h. No arsenosugars were identified in Rosetta expressing both *SsarsM* and *SsarsS*. The major arsenic species found in medium of Rosetta cells expressing both *SsarsM* and *SsarsS* or *SsarsM* alone was DMAs(V) (Figure 3). As(III) dominated in medium of Rosetta expressing *SsarsS* alone. Comparative analysis of the aqueous fraction of Rosetta cells expressing *SsarsM* alone, or *SsarsS* alone, or both *SsarsM* and *SsarsS* revealed that Rosetta cells expressing both *SsarsM* and *SsarsS* generated dimethylarsinoyl-hydroxycarboxylic acids (Figure 3B), indicating that biosynthesis of these species requires the concerted action of ArsM and ArsS. These unknown compounds were identified by HPLC-ESI-HR-MS-MS after Rosetta cells expressing both *SsarsM* and *SsarsS* were incubated with 3 μ M arsenic for 72 h, and they were a group of dimethylarsinoyl-hydroxycarboxylic acids (DMA-HA, Figures S9 and S10).

None of the dimethylarsinoyl-hydroxycarboxylic acids that cells expressing *SsarsM* and *SsarsS* together produced eluted at the same position as 5'-deoxy-5'-dimethylarsinoyl-adenosine, which is a predicted intermediate in the arsenosugar synthesis pathway proposed by Edmonds and Francesconi⁴ (Table 1). The results of HPLC-ICP-MS/ESI-MS-MS and HPLC-ESI-HR-MS-MS measurements suggested the presence of additional dimethylarsinoyl-hydroxycarboxylic acids such as 3'-dimethylarsinoyl-2'-hydroxypropionic acid, 4'-dimethylarsinoyl-2',3'-dihydroxybutyric acid, 5'-dimethylarsinoyl-2'-hydroxypentanoic acid, and 5'-dimethylarsinoyl-2',3',4'-trihydroxypentanoic acid, as well as two unidentified arsenic compounds which could not be characterized due to low concentrations (Table 1 and Figure S10). A compound with the predicted composition C₇H₁₆O₅As (*m/z* 255.02079) was also detected by HPLC-ESI-HR-MS-MS, but the structure could not be confirmed (Figure S10). Figure S10 shows that in addition to the four dimethylarsinoyl-hydroxycarboxylic acids (except 5-dimethylarsinoyl-2'-hydroxypentanoic acid) identified in incubation medium, cells expressing *SsarsM* and *SsarsS* also contained the four corresponding thio-arsenic compounds.

There are several possible reasons why dimethylarsinoyl-hydroxycarboxylic acids were produced rather than 5'-deoxy-5'-dimethylarsinoyl-adenosine in *E. coli* expressing both *SsarsM* and *SsarsS*. First, there might be additional steps catalyzed by as-yet unidentified *Synechocystis* enzymes not present in *E. coli*. Second, most of the ArsS produced in *E. coli* may be partly active, consistent with our observation that purified ArsS is extremely oxygen sensitive. Oxygen induces the switch from [4Fe-4S]⁺ to [4Fe-4S]²⁺, [3Fe-4S]²⁺, or [2Fe-2S]²⁺ with loss of biological activity. A future goal is to express the genes under anaerobic growth conditions.

Roles of Cysteine Residues in *SsarsS*.

Assuming that the production of dimethylarsinoyl-hydroxycarboxylic acids reflects a partial activity of SsArsS, the role of cysteine residues in that activity was examined. Thiol chemistry plays an important role in arsenic metabolism^{24,25} and transport processes²⁶ through formation of metalloid-sulfur bonds between arsenic and related proteins. Each of

the 11 cysteine residues in SsArsS protein, Cys⁴¹, Cys⁴⁵, Cys⁴⁸, Cys⁶⁵, Cys¹²⁹, Cys¹⁴², Cys²⁶⁴, Cys²⁷⁹, Cys³²⁰, Cys³²³, and Cys³³¹, were individually changed to serine residues. Single mutants in *SsarsS* and *SsarsM* were cotransformed into *E. coli* strain Rosetta (DE3) which were treated with As(III) (Figure S11). Three mutant proteins, C65S, C129S, and C142S, were able to produce unknown organoarsenicals. Cells expressing the other eight mutants and *SsarsM* lost the ability to produce the unidentified arsenic compounds. The three cysteine residues, Cys⁴¹, Cys⁴⁵, and Cys⁴⁸, in the N-terminal region of SsArsS correspond to the canonical radical SAM cysteine motif CX₃CX₂C, while other three cysteine residues, Cys³²⁰, Cys³²³, and Cys³³¹, in the C-terminal region of SsArsS are conserved among ArsSs.

Function of Microbial arsS Genes in Arsenic Trans-formation.

We identified putative ArsS orthologs with eight conserved cysteine residues and a radical SAM domain in other microbes. Genes encoding for ArsS from four other cyanobacteria and one eukaryotic green alga (Table S4) were coexpressed with *SsarsM* in *E. coli*. The cyanobacteria *Nostoc* sp. PCC 7120^{18,27} and the eukaryotic alga *Chlorella*²⁸ have been reported to produce arsenosugars. Recently, a putative ArsM was identified in the picocyanobacterium *P. marinus* str. MIT 9313,²⁹ although it has not been demonstrated if this species generates arsenosugars. Cyanobacteria *A. platensis* NIES-39 and *S. elongatus* PCC 6301, which have genes encoding ArsS orthologs were chosen as representative cyanobacteria. Arsenic transformation was analyzed in cells of *E. coli* coexpressing genes for orthologs of SsArsS in pETDuet-*arsS* together with pET-28a-*SsarsM*. *E. coli* coexpressing *SsarsM* and *NsarsS* from *Nostoc* sp. PCC 7120 produced the dimethylarsinoyl-hydroxycarboxylic acids observed in *E. coli* expressing *SsarsM* and *SsarsS* (Figure 4 and Figure S6B). ArsSs from cyanobacteria *A. platensis* NIES-39, *S. elongatus* PCC 6301, and *P. marinus* str. MIT 9313, organisms that have not been previously reported to generate arsenosugars, also synthesized dimethylarsinoyl-hydroxycarboxylic acids (Figure 4). Only SsArsM and CvArsS from the eukaryotic algae *C. variabilis* did not synthesize 5'-dimethylarsenoriboside derivatives when coexpressed in *E. coli*. The reason for this negative result is unknown, but perhaps CvArsS, which undergoes extensive post-translational processing in *C. variabilis*, is not modified to an active enzyme in *E. coli*, or perhaps those gene products did not fold properly in *E. coli*.

DISCUSSION

The most abundant arsenic compounds identified in marine algae are arsenosugars.³⁰ Prokaryotic cyanobacteria,²⁷ eukaryotic green algae^{28,31} and fungi all have been reported to produce arsenosugars.³² Arsenosugars have also been found in underwater plants as well;³³ their source, however, is likely to be epiphytic algae or bacteria because plants cannot methylate arsenic,³⁴ and dimethylarsenic has been postulated to be the immediate precursor of arsenosugars.¹¹ Moreover, the physiological role of arsenosugars is still unclear. For the oxo-arsenosugars, it is known to be nontoxic to humans, one arsenosugar (Oxo-Gly) was shown to be nontoxic to murine peritoneal macrophages and alveolar macrophages at micromolar levels.³⁵ The toxicity of thio-arsenosugars whose bioaccessibility are much higher than their oxo-analogs, and trimethylated arsenosugars have not been proven yet.

Arsenosugar biosynthesis may serve both as a detoxification process for the organisms which generate them, and as a precursor for more complex organoarsenicals. Unraveling the mechanism of arsenosugar biosynthesis will help determine toxicity of arsenosugars to human and the physiological role of arsenosugars in arsenic biogeochemical cycling.

Until our study, the pathway of arsenosugar biosynthesis was unknown. Edmonds and Francesconi⁴ proposed a pathway in marine alga in which DMAs(V) is reduced to DMAs(III), which is then oxidized by addition of the deoxyadenosyl group from SAM to yield the intermediate 5'-deoxy-5'-dimethylarsino-adenosine. Enzymatic, hydrolytic removal of adenine would be followed by formation of arsenosugars. This proposal is supported by the identification of the proposed intermediate 5'-deoxy-5'-dimethylarsino-adenosine in the kidney of the giant clam *Tridacna maxima*,³⁶ which contains large quantities of arsenosugars owing to symbiotic algae growing in the mantle of the clam. However, the product of arsenic methylation by ArsM enzymes is almost certainly DMAs(III) and not DMAs(V),^{37,38} so reduction of DMAs(V) is not required. The ArsM reaction is required for producing the substrate of ArsS, but DMAs has other fates such as further methylation to TMAs, so it is not a committed step in the reaction pathway. Moreover, we show that coexpression of ArsM and ArsS in *E. coli* produced many dimethylarsino- hydroxycarboxylic acids but not 5'-deoxy-5'-dimethylarsino-adenosine. We are considering two possible pathways for arsenosugar biosynthesis to account for the compounds produced by *E. coli*. Regardless of the actual pathway, our data strongly support our hypothesis that the ArsS reaction catalyzes the first committed step in arsenosugar biosynthesis.

The likely reason that arsenosugars are not generated by *E. coli* coexpressing *SsarsS* and *SsarsM* is that the intermediate of arsenosugar biosynthesis cannot be further transformed into arsenosugars because *E. coli* does not have the genes involved in arsenosugar biosynthesis. An alternative fate for this intermediate, however, could be that it is rapidly degraded in vivo to small arsenic-containing compounds such as those that we found produced by the transgenic *E. coli* constructed for this study.

It is notable that the *Synechocystis* *ars* operon containing *arsM* and *arsS* is not induced by As(III) or As(V), and the operon does not contain an *arsR* gene for an As(III)-responsive transcription factor.³⁹ Consistent with our observation of constitutive expression of the *arsM* and *arsS* genes, the amount of arsenosugars and arsenosugar phospholipids in *Synechocystis* treated with different concentrations of As(V) did not significantly increase with increasing arsenic concentrations.¹⁷ The greater accumulation in *Synechocystis* of arsenosugar phospholipid compared with arsenosugars suggests that arsenosugars are the precursors of arsenosugar phospholipids and not the end products. The absence of arsenosugar intermediates in *Synechocystis* suggests that DMAs is rapidly transformed into arsenosugars in those cells, and that the arsenosugar intermediates, DMAs(III) and MAs(III), are probably transient intermediates that do not accumulate in the cells.

Thus, we hypothesize that SsArsS, a putative radical SAM enzyme, adds a 5'-deoxyadenosyl radical (dAdo•) to DMAs-(III) and hydrolyzes the adenine from 5'-deoxyadenosine simultaneously to generate 5'-deoxy-5'-dimethylarsinoyl-ribose. SsArsS has a CX₃CX₂C motif similar to the assembly site for the [4Fe-4S] cluster found in radical

SAM enzymes. In addition, SsArsS has the other three cysteine residues in the C-terminal region of SsArsS, Cys320, Cys323, and Cys331, a CX₂CX₇C motif that binds another putative [4Fe-4S] cluster (Figure S12), which would put it in a subgroup of radical SAM enzymes that bind two [4Fe-4S] clusters, including MoaA,²⁰ BioB,⁴⁰ FbiC,⁴¹ LipA,⁴² and TYW1.⁴³ However, this CX₂CX₇C motif in the C-terminal region of the SsArsS protein, which is not conserved in all radical SAM enzymes, is 5'-deoxy-5'-not similar to other proteins that contained two or more iron-sulfur clusters.¹⁹ Mutations in any of the cysteine residues in the CX₂CX₇C motif resulted in the loss of the function for arsenosugar synthesis, consistent with a role for that motif in ArsS function. Moreover, none of the cysteine mutants in either motif produced 5'-deoxy-5'-dimethylarsinoyl-adenosine (Figure S11), indicating that the 5'-deoxy-5'-dimethylarsinoyl-ribose derivative was formed before 5'-deoxy-5'-dimethylarsinoyl-adenosine.

In summary, ArsS is the only member of the radical SAM superfamily shown to be involved in arsenosugar synthesis. This study adds a new function to the repertoire of enzymes of organoarsenical biotransformations and the radical SAM superfamily. Further biochemical analysis of complex arsenic species and their molecular mechanisms of biosynthesis and degradation will enable a better understanding for the ecological roles of these complex organoarsenicals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Cyanobacterial *ars* operons. Shown are the putative operons of *Synechocystis* sp. PCC 6803 and *Nostoc* sp. PCC 7120. The *arsM* (*slr0303*, *alr3095*) genes encode As(III) *S*-adenosylmethionine methyltransferases. *Slr0305* from *Synechocystis* sp. PCC 6803 belongs to SNARE-associated superfamily, *alr3096* of *Nostoc* sp. PCC 7120 encodes PitA (the low affinity inorganic phosphate transporter), *arsS* (*slr0304*, *alr3097*) encodes a radical SAM superfamily enzyme shown in this report to be involved in arsenosugar biosynthesis.

Figure 2.

Anion-exchange HPLC-ICP-MS chromatograms of arsenic species from (a) wild-type *Synechocystis* and (b) *Synechocystis arsS* after exposure to 1 μ M As(III) for 2 weeks, (c) wild-type *Synechocystis* and (d) *Synechocystis arsS* incubated with 10 μ M MAs(V) for 2 weeks, (e) wild-type *Synechocystis*, and (f) *Synechocystis arsS* treated with 100 μ M DMAs(V) for 1 week.

Figure 3.

A. Western blot analysis of SsArsM and/or SsArsS expression in *E. coli* strain Rosetta bearing the indicated expression plasmid constructs. MSR, MR, SR, or CK containing pETDuet-*SsarsS* and pET-28a-*SsarsM*, pET-28a-*SsarsM*, pETDuet-*SsarsS*, or pETDuet and pET-28a, respectively, induced by IPTG. B. Typical chromatograms of arsenic species in *E. coli* strain Rosetta medium incubated with 3 μ M As(III) by ion-pairing reverse-phase column HPLC-ICP-MS. DMA-HA indicates the dimethylarsinoyl-hydroxycarboxylic acids.

Figure 4.

Arsenic species transformed by different combination of ArsS orthologs and SsArsM. A. Western blot analysis of SsArsM and different ArsS orthologs expression in Rosetta bearing the indicated expression plasmid constructs. MNsS, MSeS, MPmS, MApS, and MCvS indicate *E. coli* strain Rosetta cells containing pET-28a-*SsarsM* and pETDuet-*NsarsS*, pETDuet-*SearsS*, pETDuet-*PmarsS*, pETDuet-*AparsS*, or pETDuet-*CvarsS* induced by IPTG, respectively. B. Typical chromatograms of arsenic analysis in *E. coli* strain Rosetta medium treated with As(III) by ion-pairing reversed-phase column HPLC-ICP-MS of SsArsM and different ArsS orthologs expressed in *E. coli* strain Rosetta. DMA-HA indicates the dimethylarsinoyl-hydroxycarboxylic acids. The mix consists of As(III), MAs(V), DMAs(V), As(V), and TMAO.

Arsenic Species Determined by HPLC-ICP-MS/HR-ESI-MS-MS in *E. coli* Cells and Medium When Expressing *SsarsM* and *SsarsS* (Induced With 0.5 mM IPTG and 3 μ M As(III) or As(V))

Table 1.

	m/z	Formula	Delta m [ppm]	Molecular structure
A	138.97346	C ₂ H ₈ O ₇ As	0.02

	m/z	Formula	Delta m [ppm]	Molecular structure
B	210.99459	C ₃ H ₁₂ O ₄ As	-0.41
C	239.02589	C ₇ H ₁₆ O ₄ As	-0.99

	m/z	Formula	Delta m [ppm]	Molecular structure
D	241.00514	C ₆ H ₁₄ O ₅ As	-1.00
E	271.0157	C ₇ H ₁₆ O ₆ As	-0.87