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Functional characterization of the methylarsenite-inducible *arsRM* operon from *Noviherbaspirillum denitrificans* HC18

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Summary:

Microbial arsenic methylation by arsenite (As(III)) *S*-adenosylmethionine methyltransferases (ArsMs) can produce the intermediate methylarsenite (MAs(III)), which is highly toxic and is used by some microbes as an antibiotic. Other microbes have evolved mechanisms to detoxify MAs(III). In this study, an *arsRM* operon was identified in the genome of an MAs(III)-methylation strain *Noviherbaspirillum denitrificans* HC18. The *arsM* gene (*NdarsM*) is located downstream of an open reading frame encoding an MAs(III)-responsive transcriptional regulator (NdArsR). The *N. denitrificans arsRM* genes are co-transcribed whose expression is significantly induced by MAs(III), likely by alleviating the repressive effect of ArsR on *arsRM* transcription. Both *in vivo* and *in vitro* assays showed that NdArsM methylates MAs(III) to dimethyl- and trimethyl-arsenicals but does not methylate As(III). Heterologous expression of *NdarsM* in arsenic-sensitive *Escherichia coli* AW3110 conferred resistance to MAs(III) but not As(III). NdArsM has the four conserved cysteine residues present in most ArsMs, but only two of them are essential for MAs(III) methylation. The ability to methylate MAs(III) by enzymes such as NdArsM may be an evolutionary step originated from enzymes capable of methylating As(III). This finding reveals a mechanism employed by microbes such as *N. denitrificans* HC18 to detoxify MAs(III) by further methylation.

Keywords

Methylarsenite (MAs(III)); MAs(III) methylation; transcriptional regulator ArsR; As(III) S-adenosylmethionine (SAM) methyltransferases (ArsM)

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Introduction

Arsenic exists as various chemical species, both inorganic and organic in trivalent and pentavalent oxidation states. The toxicity of arsenic highly depends on its species. Methylarsenite (MAs(III)), an intermediate product of arsenic biomethylation, is more toxic and potentially carcinogenic than either the corresponding pentavalent species or inorganic arsenite (As(III)), and may account for the majority of arsenic-related diseases (Yoshinaga-Sakurai et al., 2020; Li et al., 2021; Styblo et al., 2021). MAs(III) is stable in the anoxic environment that existed before the Great Oxidation Event (GOE) (Chen et al., 2020). When the atmosphere became oxidizing after the GOE, MAs(III) was constantly oxidized by atmospheric oxygen to the much less toxic pentavalent oxidation state, methylarsenate (MAs(V)) (Fru et al., 2015). In addition, anthropogenic use of the herbicide monosodium methylarsenate provides another source of MAs(V) (Yoshinaga et al., 2011).

Microbial arsenic methylation has been proposed to play a significant role in the biogeochemical cycling of arsenicals and environmental health (Zhao et al., 2013; Zhu et al., 2014). Since the first report of bacterial arsenic methylation(Qin et al., 2006), a number of phylogenetically diverse As(III) methylating microbes have been isolated from different environments (Kuramata et al., 2015; Wang et al., 2015; Huang et al., 2016; Chen et al., 2017b; Huang et al., 2018). Methylation is catalyzed by the enzyme As(III) *S*-adenosylmethionine (SAM) methyltransferase (ArsM in microbes or AS3MT in animals) (Qin et al., 2006; Wood et al., 2006). Under oxic conditions, the trivalent products of microbial-mediated As(III) methylation are oxidized to relatively non-toxic MAs(V) and dimethylarsenate (DMAs(V)). Thus methylation is an arsenic detoxification pathway for aerobes that harbor *arsM* genes. However, under anoxic conditions, As(III)-methylating anaerobes produce highly toxic MAs(III), which is actively extruded into their surroundings, posing an environmental challenge for their neighbors without MAs(III) resistant genes (Li et al., 2016; Styblo et al., 2021). Thus, As(III) methylation is considered as an antibiotic-producing pathway in anaerobes.

Microorganisms have evolved a variety of pathways for MAs(III) resistance (Li et al., 2016), including MAs(III) oxidation (Chen et al., 2015; Zhang et al., 2021), MAs(III) demethylation (Yoshinaga and Rosen, 2014) as well as MAs(III) methylation (Dheeman et al., 2014). Additionally, MAs(III) extrusion occurs via the MAs(III) efflux permeases ArsP or ArsK (Chen et al., 2017a; Shi et al., 2018). Taken together, these microbial transformations contribute to an organoarsenical biogeochemical cycle. Genes involved in MAs(III) detoxification are usually found in arsenic resistance (*ars*) operons that are controlled by an ArsR As(III)-responsive transcriptional repressor. Although most of the ArsR repressors studied to date are responsive to As(III) (Shi et al., 1994; Qin et al., 2007; Ordonez et al., 2008), a novel ArsR of *Shewanella putrefaciens* 200 has been demonstrated to be selective for MAs(III) over As(III) and controls expression of genes for MAs(III) detoxification (Chen et al., 2017a).

Here we examined the properties of an ArsM ortholog from *Noviherbaspirillum denitrificans* HC18. *N. denitrificans* HC18 is an arsenic-resistant facultative anaerobe which uses nitrate as an electron acceptor for growth (Wu et al., 2021). The genome of *N. denitrificans* HC18

(GenBank no. GCA_003918735.1) contains a genomic island with As(III) oxidase *arx* and *aio* gene clusters, and an *arsRM* operon. In this study, the *arsRM* operon of *N. denitrificans* HC18 was shown to be responsible for MAs(III) methylation, and the function of the *arsM* gene in resistance to MAs(III) was investigated. This study contributes to our understanding of the genetics and enzymology of microbial arsenic methylation.

Result:

Arsenic transformation by *N. denitrificans* HC18

We characterized the resistance of *N. denitrificans* HC18 to MAs(III). Based on the growth data at 24 h, cell growth of HC18 was inhibited by 1 – 5 μM MAs(III) and 200 – 800 μM As(III). The concentrations of MAs(III) and As(III) that caused 50% growth inhibition (EC_{50}) were estimated to be 3.5 μM and 299 μM, respectively (Fig. S1). MAs(III) was methylated to DMAs(V) and trimethylarsine oxide (TMAs(V)O) by *N. denitrificans* HC18 during a 12 h period. During the first 6 h, the concentration of DMAs(V) increased rapidly in the culture medium, concomitant with the decrease of MAs(III) (Fig. 1). TMAs(V)O accounted for only 8–11 % of the total arsenic in the culture medium, and it appeared only after 3 h. As(III) was barely detectable in the medium during the experiment, suggesting no demethylation of MAs(III). When HC18 cells were incubated with 10 μM As(III), no methylated arsenicals were detected in the medium, and all of the arsenic was recovered as As(V) after oxidation with H_2O_2 (Fig. S2).

In silico analysis of the *arsRM* operon

By analyzing the draft genome of *N. denitrificans* HC18 (GenBank no. GCA_003918735.1), the *N. denitrificans* HC18 *arsM* (designated *NdarsM*, GenBank no. RZI40429) gene is located downstream of an open reading frame (designated *NdarsR*, GenBank no. RZI40430) encoding a protein homologous to the MAs(III)-responsive transcriptional regulator ArsR from *S. putrefaciens* 200 (Fig. 2A, Fig. S3). An additional MAs(III) detoxification mechanism consisting of an MAs(III) efflux permease ArsP (RZI44527) and an MAs(III)-selective ArsR (ArsR2, GenBank no. RZI44528) was also found in the genomic DNA (Fig. S3). Reverse transcription (RT)-PCR with cDNA as the template confirmed that *arsRM* genes are cotranscribed in the presence of 1 μM MAs(III) (Fig. 2B). A comparative analysis was carried out to evaluate expression of *NdarsR* and *NdarsM* in the presence or absence of arsenic. The RT-qPCR results showed that the transcriptional level for *NdarsR* and *NdarsM* increased 2.1 and 4.3-fold, respectively, in response to 10 μM As(III) compared to cells grown without arsenic. The transcriptional levels of *NdarsR and NdarsM* genes were upregulated by 4.4-fold and 65.7-fold, respectively, in response to 1 μM MAs(III) compared to the level of the control (Fig. 2C). These results indicate that MAs(III) is a much stronger inducer of the expression of *arsRM* operon.

Sequence analysis showed that NdArsM has a relatively high similarity with the ArsM of *Rhodopseudomonas palustris* (45.8% identity). It exhibits significant, but lower identity (30.9 – 35.9%) to the ArsMs of mammalian AS3MT methyltransferase and cyanobacterial species. Compared with other six typical orthologs, NdArsM contains four cysteines (in positions 32, 67, 155 and 205) that correspond to the four conserved cysteine residues

found in most ArsM orthologs (Fig. 3). NdArsR has a potential MAs(III) binding site composed of two conserved cysteines in the C-terminus, and does not have a cysteine residue corresponding to *Acidithiobacillus ferrivorans* ArsR (AfArsR) residue 102 (Fig. S4). This is consistent with ArsR repressors that are selective for MAs(III) and require only two coordinations for binding, while those that bind As(III) often have a third cysteine residue for three-coordinate binding (Chen et al., 2017a). To examine the evolutionary history of NdArsR-like MAs(III)-responsive repressors, a phylogenetic analysis of ArsR sequences from different bacterial species was conducted (Fig. S5). The ArsR repressors cluster in four groups. ArsRs that have a three-coordinate As(III) binding site cluster in groups I – III. MAs(III)- responsive repressors that have a two-coordinate MAs(III) binding site and control expression of MAs(III) resistance genes (*arsP*, *arsH* and *arsM)* cluster in group IV. NdArsR clusters in group IV of the ArsR family that has only two conserved cysteine residues. We propose that *N. denitrificans* HC18 responds and detoxifies environmental MAs(III) through NdArsM methylation activity regulated by an MAs(III)-selective NdArsR.

Regulation of the *ars* promoter by MAs (III)

To examine the function of NdArsR in transcription of the *arsRM* operon, an arabinose-repressible expression system (Fig. S6) was constructed that uses the reporter gene *gfp* driven by the *arsRM* promoter (P*ars*) in *E. coli* AW3110*Δars* (Carlin et al., 1995). In the absence of arabinose repressor and arsenic inducers, the pACYC184-P*ars-gfp* fusion produced a high level of fluorescence (Fig. 4A). Expression was nearly absent in the presence of arabinose alone but increased significantly with the co-addition of 3 μM MAs(III). Addition of As(III), As(V), MAs(V) or DMAs(V) gave no response (Fig. 4A). From these findings, we conclude that NdArsR serves as a transcriptional repressor of the *arsRM* operon and that MAs(III) is the preferred inducer. We hypothesize that MAs(III) binds to NdArsR, disrupting interaction between NdArsR and its DNA binding site. To test this hypothesis, the effect of addition of various concentrations of MAs(III) to the culture medium was examined. Addition of MAs(III) up to 4 μM resulted in increasing levels of fluorescence (Fig. 4B). These results are consistent with MAs(III) binding to NdArsR resulting in dissociation of the repressor from the DNA.

NdArsM methylates and confers resistance to MAs(III) but not As(III)

NdarsM expression in the *E. coli* As(III)-hypersensitive strain AW3110*Δars* conferred resistance to MAs(III) but did not complement the As(III) sensitive phenotype. In contrast, cells of *E. coli* AW3110*Δars* expressing the *CrarsM* gene from the photosynthetic alga *Chlamydomonas reinhardtii* (Chen et al., 2013) were resistant to both MAs(III) and As(III) (Fig. 5 A and B).

To elucidate whether NdArsM has MAs(III) methylation activity *in vivo*, cells of *E. coli* AW3110 expressing *NdarsM* were grown in M9 medium (Sambrook and Russel, 2011) containing 4 μM MAs(III) for 2 h. The level of MAs(III) decreased, with a concomitant increase in DMAs(V) and TMAs(V)O in the culture medium (Fig. 6A). In these experiments the reactions were terminated with H_2O_2, which oxidizes and solubilizes the products. In contrast, when cells of *E. coli* AW3110(pET29a-*NdarsM*) were incubated with 4 μM As(III), all of the arsenic in the culture medium was recovered as As(V) after H_2O_2 treatment. As a

positive control, cells expressing *CrarsM* methylated either As(III) or MAs(III) to DMAs(V) and TMAs(V)O within 2 h (Fig. 6B). These results are consistent with NdArsM methylating MAs(III) but not As(III).

Purified NdArsM exhibits a molecular mass of approximately 28 kDa, which is consistent with its predicted molecular mass (Fig. S7). To examine the mechanism of the enzyme in more detail, biotransformation of arsenic species *in vitro* was assayed with purified NdArsM over 2 h. NdArsM methylated MAs(III) to DMAs(V) and TMAs(V)O in a time-dependent manner, but As(III) methylation was not observed (Table 1). These results demonstrate that the preferred substrate of NdArsM is MAs(III), not As(III).

Role of cysteine residues in MAs(III) methylation

There are four conserved cysteine residues (Cys32, Cys67, Cys155, and Cys205) in NdArsM (Fig. 3). To investigate the role of these cysteine residues in MAs(III) methylation, each was individually altered to a serine residue. Each of the four conserved cysteine mutant proteins (C32S, C67S, C155S, and C205S) were purified and assayed for MAs(III) methylation activity. Purified derivatives C32S and C67S exhibited similar MAs(III) methylation activity as wild type NdArsM, but neither the C155S nor the C205S enzymes exhibited measurable MAs(III) methylation activity. These results support a role for two conserved cysteine residues, Cys155 and Cys205, in MAs(III) methylation (Fig. S8).

In silico docking of MAs(III) to NdArsM

Because the structures of CmArsM (ArsM from *Cyanidioschyzon* sp. 5508, PDB ID: 4FSD) with or without ligands (As(III) and phenylarsine oxide (PAO)) are available (Ajees et al., 2012; Marapakala et al., 2015), we use unliganded CmArsM as a template for NdArsM homology modelling. MAs(III) was docked *in silico* to the homology mode. The MAs(III) molecule is bound at the conserved arsenic binding site. The arsenic atom is at a distance of 2.59 Å from the sulfur atom of conserved cysteine residue Cys205 and 3.96 Å from the sulfur atom of conserved cysteine residue Cys155 (Fig. S9A and B). While the homology model clearly demonstrates how NdArsM could bind MAs(III) two-coordinately to conserved cysteine residues 155 and 205, it does not explain the specificity for MAs(III) over As(III). In CmArsM, As(III) is bound three-coordinately to Cys174 and Cys224, which are the equivalent of NdArsM Cys155 and Cys205, and CmArsM residue Cys44 contributes the third sulfur ligand (Packianathan et al., 2018). In NdArsM the residue corresponding to CmArsM Cys44 is Cys32, which is not visible in the NdArsM homology model. The C32S mutant still methylates MAs(III) (Fig. S8), showing that Cys32 is not required for MAs(III) binding. Since NdArsM does not methylate As(III), Cys32 is probably not a third ligand even though it cannot be seen in the homology model.

A major difference between NdArsM and CmArsM is that NdArsM lacks the conserved C-terminal domain found in most ArsM/AS3MT enzymes.The lack of the C-terminal domain in NdArsM is evident from superimposition of the two structures (Fig. S9C). This difference may be the reason why CmArsM can methylate As(III), whereas NdArsM cannot. To test this hypothesis, the C-terminal region (amino acids 283 to 401) in CmArsM was deleted. Both wild type CmArsM and truncated CmArsM protein were expressed in *E. coli*

BL21(DE3). The truncated CmArsM protein was still able to catalyze As(III) methylation as the wild type CmArsM (Fig. S10). These results indicates that the C-terminal domain is not required for As(III) binding and lacking the conserved C-terminal domain is not the reason for the lack of As(III)-methylating activity in NdArsM.

Discussion

This study describes the contribution of the soil bacterium strain *N. denitrification* HC18 to the organoarsenical biogeochemical cycle. *N. denitrification* HC18 is not able to oxidize As(III) to As(V) nor methylate As(III) under oxic conditions but can methylate MAs(III) to DMAs(V) and TMAs(V)O. *N. denitrification* HC18 is not able to oxidize MAs(III) to MAs(V) nor demethylate MAs(III) under oxic conditions. When the *NdarsM* gene was heterologously expressed in the arsenic-sensitive strain *E. coli* AW3110, the cells became MAs(III) tolerant. In addition, purified NdArsM was shown to catalyze methylation of MAs(III) to DMAs(V) and TMAs(V)O. However, NdArsM was unable to methylate inorganic As(III), nor did the *NdarsM* gene confer resistance to As(III). Our results clearly show that NdArsM is the primary determinant of MAs(III) detoxification in *N. denitrification* HC18. To date, a number of soil microbes have been demonstrated to produce MAs(III), either by MAs(V) reduction (Yoshinaga et al., 2011) or by methylation of As(III) (Qin et al., 2006). MAs(III) is highly toxic and has been shown to have antibiotic activity (Li et al., 2016; Styblo et al., 2021). Further methylation of MAs(III) to di- and trimethyl trivalent species may be protective if those species are rapidly oxidized or volatilized. We propose that the role of MAs(III)-specific NdArsM is to protect against the toxicity of MAs(III) generated by MAs(III) producers.

ArsM is widely distributed in the members of every kingdom from bacteria to human. Phylogenic analysis suggests that *arsM* genes have been horizontally transferred to diverse kingdoms in at least six distinct events, likely to allow adaptation of life to the presence of arsenic compounds in the environment (Chen et al., 2017c). Throughout its evolutionary history, ArsM proteins exhbit different arsenic methylation properties. For example, some ArsMs such as those in *Rhodopseudomonas palustris* and *Arsenicibacter rosenii* can methylate As(III) efficiently to produce mainly trimethylated arsenicals (Qin et al., 2006; Huang et al., 2016), whereas some other ArsMs show low methylation activities and rarely produce trimethylated arsenicals (Zhang et al., 2015; Huang et al., 2018; Pedersen et al., 2020). Molecular clock analyses of microbial gene families involved in arsenic transformation and detoxification suggest that arsenic methylation emerged earliest in the ancient Archean environment well before the Earth became oxygenated (Chen et al., 2020). The earliest microbial ArsMs are likely involved in the methylation of As(III) as a detoxification mechanism (Chen et al., 2017c). However, some microbes appear to have used MAs(III) as an primitive antibiotic to gain a competitive advantage against other microbes (Li et al., 2016; Li et al., 2021). Facing the challenge of MAs(III), some microbes have evolved different strategies to detoxify MAs(III). One of the strategies is to methylate MAs(III) into relatively nontoxic DMAs(V), such as that employed by *N. denitrificans* HC18. We postulate that NdArsM may originate from As(III)-methylating ArsMs, because there is some similarity in the protein sequences between NdArsM and other As(III)-methylating ArsMs (30.9 – 45.8%) and logically MAs(III)-methylating ArsMs should

emerge after As(III)-methylating ArsMs. The reason why NdArsM does not methylate As(III) is not clear, as all conserved cysteine residues required for As(III) methylation are present and missing the C-terminus domain does not appear to affect As(III) methylation. Further studies are needed to identify the structural features of NdArsM governing the substrate specificity.

Similar to the genes in other arsenic methylating bacteria, *NdarsM* is located in an *ars* operon downstream of an MAs(III)-selective *arsR* transcriptional repressor. NdArsR has a two cysteine-arsenic binding motif for binding of MAs(III) (Chen et al., 2017a) and regulates *NdarsM* transcription. Transcription of *NdarsM* is derepressed by MAs(III) but not As(III). This is consistent with a role of NdArsM in resistance to MAs(III). Furthermore, we found that an *arsRM* operon similar to that in *N. denitrification* HC18 is also present in *Raineyella* sp. CBA3103 and *Sulfuricella denitrificans* skB26 (Fig. S11), suggesting that the MAs(III) detoxification system may be widespread. These data suggest that the MAs(III)-selective ArsR-regulated MAs(III)-methylation system has been selected by some bacteria as a general strategy for MAs(III)-detoxification.

In short, *N. denitrification* HC18 has evolved an *arsRM* operon that functions specifically to detoxify MAs(III) by methylation. Because MAs(III) is considered a primitive antibiotic, MAs(III) methylation enhances survival of *N. denitrification* HC18 in environments containing MAs(III).

Experimental procedures

Chemicals

Arsenicals and *S*-adenosyl-L-methionine (SAM) were purchased from Sigma-Aldrich. MAs(V) was obtained from Chem Service, Inc., West Chester, PA. MAs(III) was prepared as described (Reay and Asher, 1977). All reagents were analytical grade.

Bacterial strains, plasmids, primers, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table S1, and the primers used are listed in Table S2. *N. denitrificans* HC18 was cultured aerobically at 30 °C in TY medium (Beringer, 1974). Growth was determined from the A_{600nm}. *Escherichia coli* Top10 was used for plasmid construction and replication. *E. coli* AW3110(DE3) was used for arsenic resistance assays and analysis of *NdarsM* function. *E. coli* BL21(DE3) was used for protein expression. For most experiments, cultures of *E. coli* strain bearing the indicated plasmids were grown aerobically in lysogeny broth (LB) or M9 media (Sambrook and Russel, 2011) containing 0.2% glycerol at 37 °C. Antibiotics were used to supplement the medium at a final concentration of 50 μg mL^{-1} of kanamycin, 34 μg mL^{-1} of chloramphenicol, or 50 μg mL^{-1} of ampicillin.

Arsenic transformation by *N. denitrification* HC18

N. denitrificans HC18 was cultured in TY medium at 30 °C with shaking at 200 rpm, then the cells were harvested at the late exponential growth phase (A_{600nm} = 0.5) by centrifugation (2,500 × *g* for 5 min at 4 °C) and washed twice with deionized distilled water.

The washed cells were then incubated at 30 °C in TY medium with an initial cell density of A_{600nm} = 1.0, As(III) or MAs(III) were individually added into the medium at 10 μM or 1 μM. Then 0.5 mL samples were withdrawn every 3 h and acidified with 1% concentrated HCl. Each sample was then centrifuged at 10,000 × *g* at 4 °C for 10 min, and the supernatant solution was passed through a 0.22-μm membrane filter. The concentrations of arsenicals in the solution phase were analyzed by using high performance liquid chromatography coupled with inductively coupled plasma mass spectrometry (HPLC-ICP-MS) analysis.

Intergenic PCR and qRT-PCR

To evaluate the effect of arsenicals on expression of *NdarsR* and *NdarsM* genes, *N. denitrificans* HC18 was grown in TY medium at 30 °C to A_{600nm} = 0.2, and then induced with 10 μM As(III), or 1 μM MAs(III) for 8 h. The cells were harvested by centrifugation at 6,000 × *g* for 10 min. Total RNA was extracted using a bacterial RNA isolation kit (OMEGA), and genomic DNA (gDNA) was removed with DNase I at 42 °C for 2 min. RNA concentrations were quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific). Reverse transcription (RT) was conducted with a HiScript Reverse Transcriptase kit (Vazyme) with 1 μg RNA using random primers. Standard PCR was carried out using Q5 high-fidelity DNA polymerase (NEB), with primers RT-arsR-F1 and RT-arsM-R1, with cDNA as the template, RNA as the negative control, and genomic DNA from *N. denitrificans* HC18 as the positive control.

Real-time qPCR was performed using the SYBR PrimeScript RT-PCR kit (TaKaRa) on a LightCycler (version 1.5) thermocycler. The primers used for qPCR are listed in Table S2. Primer specificity was determined by examination of the melting curve at the end of amplification. All reactions were performed in triplicate in three independent experiments. Relative expression levels were estimated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), and the transcription of the house-keeping gene *gyrB* (encoding DNA gyrase subunit B) was set as a reference for normalization.

Transcription analysis

Transcriptional activity of the biosensor was estimated from arsenical responsiveness expression of a *gfp* gene under control of the *ars* promoter. A fragment comprising the putative promoter regions of the *arsRM* operon and 48 bp downstream of the initiation codons of *arsR* was amplified with the PCR primers NdPars-F and NdPars-R. The PCR product was then cloned into the *BamH*I and *Sal*I sites of the *gfp* reporter plasmid pACYC184-gfp, to generate pACYC184-Pars-gfp (Table. S1). The *NdarsR* gene from *N. denitrificans* HC18 was codon optimized for *E. coli e*xpression and chemically synthesized by Sangon Biotech (Shanghai) Co., Ltd. The gene was then inserted into the pBAD-myc-HisA expression vector using *Nco*I and *Hind*III restriction sites to generate pBAD-*NdarsR* (Table. S1). Plasmid pACYC184-Pars-gfp was electroporated into cells of *E. coli* AW3110, together with the compatible plasmid pBAD-*NdarsR*. In the presence of glycerol and the absence of arabinose, AraC is a negative regulator that represses expression of genes behind p_{ara}, in this case, of *NdarsR*, resulting in *gfp* expression (Fig. S6C). For induction assays, cultures of transformants were grown to mid-exponential phase in M9 medium at 37 °C with 100 μg mL^{-1} ampicillin and 34 μg mL^{-1} chloramphenicol with shaking. Glycerol (0.5

%) was added for constitutive expression of *gfp*. *NdarsR* gene was induced by addition of 0.2% arabinose for 5 h. Derepression was produced by simultaneous addition of arabinose and arsenicals for 5 h. Cell densities were normalized by dilution or suspension to the same A_{600nm}, and expression of *gfp* was assayed from the fluorescence of cells using a Photon Technology International spectrofluorometer with an excitation wavelength of 470 nm and emission wavelength of 510 nm.

Cloning, expression, and purification of NdArsM

Genomic DNA from *N. denitrificans* HC18 was extracted with phenol-chloroform (Sambrook and Russel, 2011). The *NdarsM* gene was amplified from genomic DNA from strain HC18 using the primer pair NdarsM-F and NdarsM-R. The PCR product digested with *Nde*I and *Xho*I was inserted into the corresponding sites of vector plasmid pET29a(+) under control of T7 promoter. Subsequently, plasmid pET29a-*NdarsM* was transformed into *E. coli* BL21(DE3) or AW3110*Δars*. Because there are three domains in the ArsM from *Chlamydomonas reinhardtii* (CrArsM) (Packianathan et al., 2014), and CrArsM was able to catalyze As(III) methylation. The *CrarsM* gene in pET29a(+) was used as a positive control (Chen et al., 2013).

To induce expression of NdArsM, cells of BL21(DE3) bearing plasmid pET29a-*NdarsM* were grown at 37 °C in LB medium containing 50 mg mL^{-1} of kanamycin with shaking at 200 rpm. At a cell density of A_{600nm} ~0.5, 0.3 mM, IPTG was added to induce expression of NdArsM. The cells were grown at 37 °C for another 12 h, harvested by centrifugation (5,000 × *g*) for 15 min, and suspended in buffer A (20% (w/v) glycerol, 20 mM imidazole, 50 mM MOPS, pH 7.5, 0.5 M NaCl and 10 mM 2-mercaptoethanol). Cells were disrupted by sonication. Cell debris were removed by centrifugation at 150,000 × *g* for 1 h, and the supernatant was loaded onto a Ni(II)-NTA column (GE Healthcare). Proteins were eluted with with buffer A plus 5 mM dithiothreitol (DTT) and 2 mM ethylenediamine tetraacetate (EDTA). Purified protein was analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970), and protein concentrations were determined using the Bradford assay (Bradford, 1976). Fractions containing NdArsM were concentrated by centrifugation using a 10-kDa cutoff Amicon Ultrafilter (Millipore, USA). After washing with buffer containing 10 mM Tris, pH 7.5, and 0.1 M KCl, proteins were buffer exchanged with buffer A lacking imidazole for storage at −80 °C until use. The yield from 1 L of cell culture was 21 mg of purified protein.

Mutagenesis of the *NdarsM* gene

The *NdarsM* gene was mutated using the QuikChange site-directed mutagenesis protocol (Agilent) with the primers containing the mutated sequences in Table S2. The sequences of the *NdarsM* mutants were confirmed by DNA sequencing. Residues Cys32, Cys67, Cys155, and Cys205 in NdArsM were changed to serine. The mutant NdArsM protein was expressed and purified by the same procedure described for wild-type NdArsM.

As(III) methylation of *CmArsM* truncated derivative *in Vitro*

The complete *CmarsM* gene from *Cyanidioschyzon* sp. 5508 was codon optimized and chemically synthesized by Sangon Biotech (Shanghai) Co., Ltd, and also cloned into

overexpression vector pET-29a(+). To construct the truncated *CmarsM* gene lacking the C terminus domain, PCR amplification was performed using the primer pair $CmarsM_{282}$-F and $CmarsM_{282}$-R (Table S2), resulting in deletion of bases from positions 847 to 1203 at the 3' end. The PCR fragment was then cloned into vector pET-29a(+) using the *Nde*I and *Xho*I restriction sites introduced by PCR. The truncated $CmArsM_{282}$ protein was expressed and purified by the same procedure described above.

Arsenic tolerance and transformation assays using recombinant *E. coli* cells.

For arsenic resistance and methylation assays, cells of *E. coli* strains AW3110 bearing plasmid pET29a-*NdarsM*, pET29a-*CrarsM* or vector plasmid pET29a were grown overnight with shaking at 37 °C in M9 medium with 50 μg mL^{-1} kanamycin. Overnight cultures were diluted 100-fold into M9 medium containing various concentrations of the indicated arsenicals induced with 0.3 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubated at 37 °C with shaking at 200 rpm for an additional 12 h. Bacterial growth was determined from the A_{600nm}. The cell culture samples were centrifuged at 10,000 × *g* for 5 m. Arsenic species were analyzed by HPLC-ICP-MS.

Assay of arsenic methylation activity

Methylation of As(III) and MAs(III) was assayed by purified NdArsM as described previously (Dheeman et al., 2014). Methylation activity of purified NdArsM was assayed at 37 °C in buffer consisting of 50 mM MOPS, pH 7.5, containing 0.3 M NaCl, 8 mM GSH and 1 mM SAM. The reactions were terminated by adding 6 % (v/v) H_2O_2 and heated at 80 °C for 5 min to oxidize all arsenic species. Denatured protein was removed by centrifugation using a 3 kDa cutoff Amicon ultrafilter. The filtrate was speciated by HPLC-ICP-MS. Where noted, H_2O_2 was not added to allow for determination of trivalent arsenicals.

Arsenic was speciated by high performance liquid chromatography (HPLC) (PerkinElmer Series 2000) using a C18 reversed-phase column eluted with a mobile phase consisting of 3 mM malonic acid, 5 mM tetrabutylammonium hydroxide, and 5 % (v/v) methanol (pH 5.6) with a flow rate of 1 mL min^{-1}, and the concentration of arsenic was determined by inductively coupled plasma mass spectrometry (ICP-MS).

Bioinformatics and homology modeling of NdArsM

The homologous sequence and conserved domain were identified using the BLASTp software provided by the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple alignment analyses of the deduced amino acid sequences were performed by Clustal_W2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Phylogenetic analysis used the neighbor-joining analysis method using MEGA 7.0 software (Kumar et al., 2016). The NdArsM homology model built on the unlignaded structure of *Cyanidioschyzon merolae* (CmArsM) (PDB ID: 4FSD) structure has been deposited in the Protein data bank. The model for NdArsM was built using a fully automated protein structure homology modeling server SWISS-MODEL (http://swissmodel.expasy.org/) (Kiefer et al., 2009). The model quality was estimated based on the QMEAN scoring function 0.39 which is within the acceptable range (Benkert et al.,

2011). PyMOL v1.3 was used to visualize the structural models (DeLano, 2001; Peters et al., 2006).

Statistical analysis.

Statistical analysis was performed with SPSS version 20.0 software. One-way analysis of variance (ANOVA) was used to calculate *P* values for the gene transcription analyses. *P* values less than 0.01 were considered statistically highly significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations:

MAs(III)	methylarsenite
MAs(V)	methylarsenate
DMAs(V)	dimethylarsenate
TMAs(V)O	trimethylarsine oxide
HPLC	high performance liquid chromatography
ICP-MS	inductively coupled plasma mass spectroscopy

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Originality-Significance Statement

Methylarsenite (MAs(III)) is an intermediate of arsenic methylation. Its high toxicity is used by some microbes as a potent antibiotic. Other microbes have evolved different mechanisms to detoxify MAs(III). This study identifies an MAs(III) selective *arsRM* operon in bacterial strain *Noviherbaspirillum denitrificans* HC18 that detoxifies MAs(III). ArsM catalyzes methylation of MAs(III) but not As(III) and its transcriptional regulator ArsR is selective for MAs(III). These results demonstrate methylation as a detoxification mechanism for the antibiotic MAs(III).

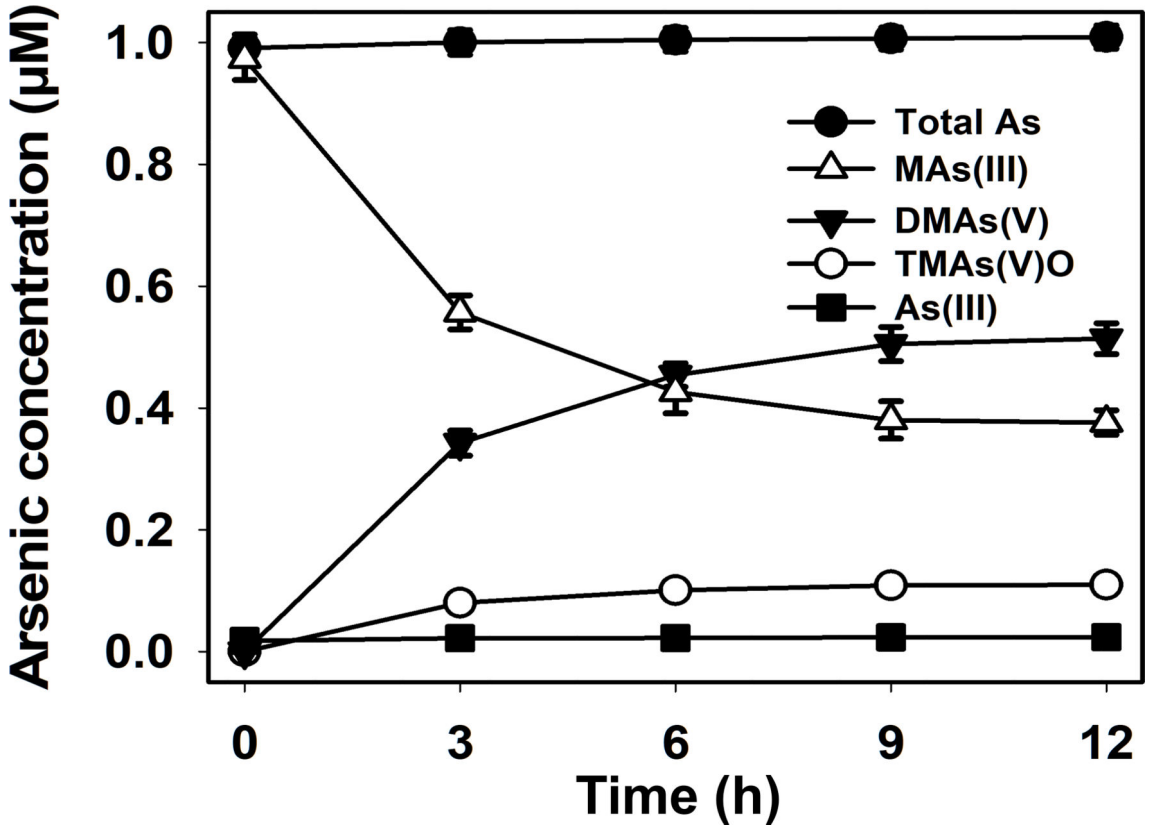

Fig. 1. Time course of MAs(III) transformation by *Noviherbaspirillum denitrificans* HC18.

N. denitrificans HC18 was grown in TY medium with 1 μM MAs(III) for 12 h at 30 °C with shaking at 200 rpm. Error bars represent standard errors (SE) from three independent assays.

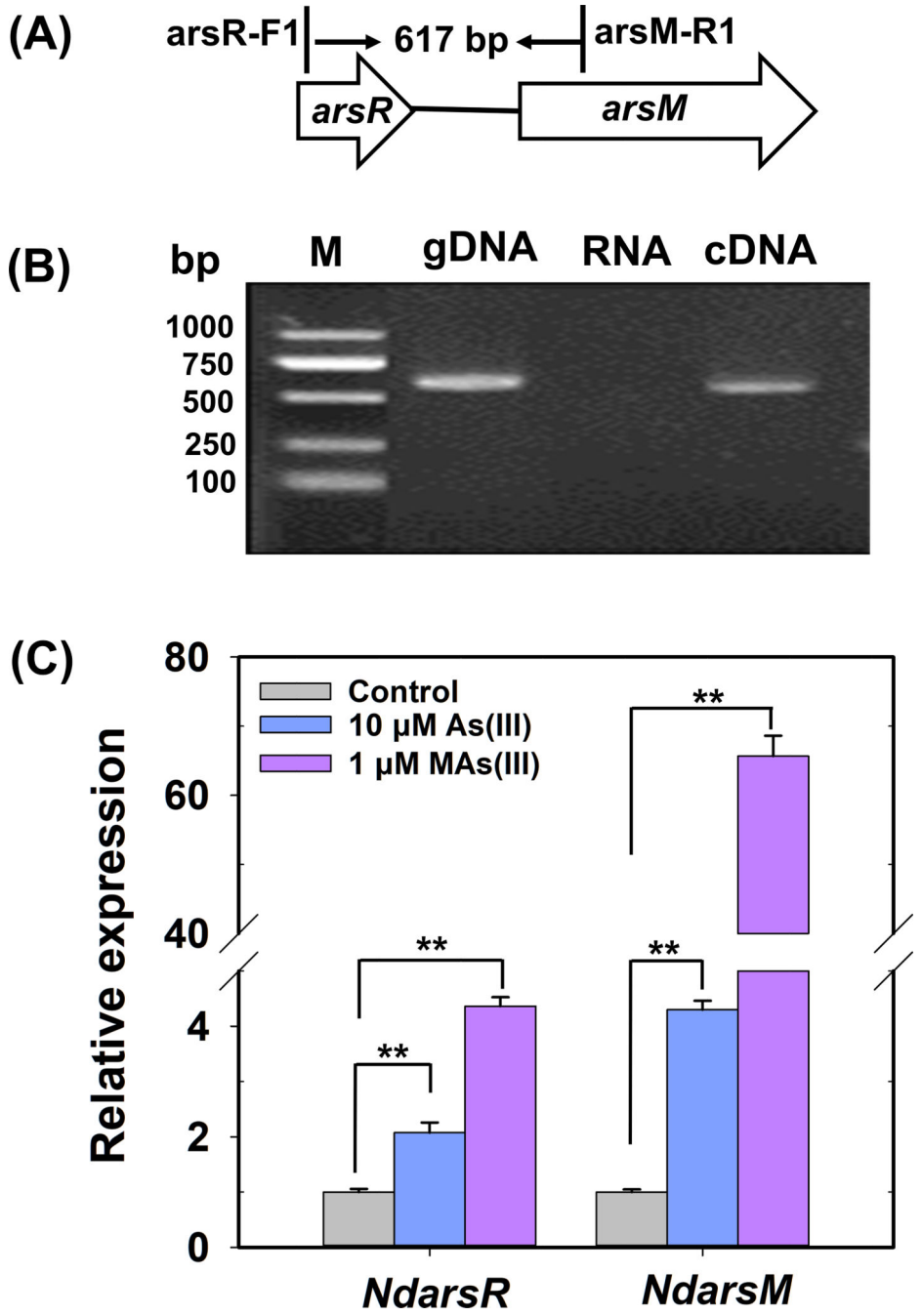

Fig. 2. Transcriptional response of the *arsRM* gene cluster to As(III) or MAs(III).

(A) Diagram of the of *ars* genes and the primers for RT-PCR analysis in *Noviherbaspirillum denitrificans* HC18. (B) RT-PCR assays to verify the cotranscription of *arsR* and *arsM*. The mRNA molecules covering the intergenic sequences of *arsR* and *arsM* genes were amplified by RT-PCR by using genomic DNA (gDNA), cDNA and RNA of strain HC18 as the template; M, DNA marker. (C)Transcriptional analysis of *arsR* and *arsM* genes in response to 1 μM MAs(III) or 10 μM As(III). Error bars represent standard errors (SE) from three independent assays. **, $p < 0.01$.

Fig. 3. Multiple sequence alignment of ArsM homologues.

ArsM from *Noviherbaspirillum denitrificans* (NdArsM, RZI40429) was aligned with homologues from *Nostoc* sp. PCC7120 (NsArsM, HQ891147), *Rhodopseudomonas palustris* (RpArsM, WP_011159102), human (hAS3MT, AAI19639), rat (rAS3MT, EDL94361), *Chlamydomonas reinhardtii* (CrArsM, AFS88933), and *Cyanidioschyzon merolae* (CmArsM, ACN39191) (GenBank accession numbers in parentheses). Underlining, triangles, cysteine residues that are probably involved in arsenical binding.

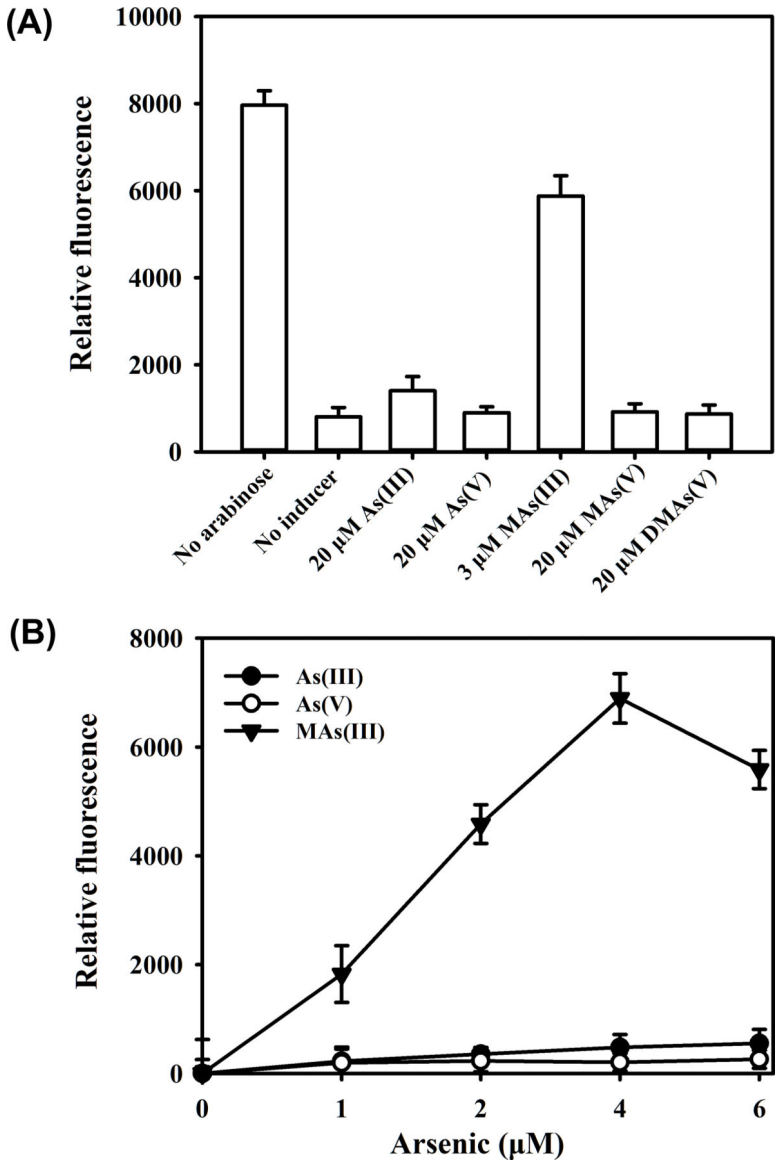

Fig. 4. Role of NdarsR in transcription regulation of the *arsRM* operon.

Cultures of *E. coli* AW3110 harboring plasmids pBAD-*NdarsR* and pACYC184-Pars-gfp were incubated for 5 h in the absence of arabinose or with 0.2% arabinose and the indicated concentrations of inducers (A) and various concentrations of arsenicals (B), following which GFP fluorescence was determined. Data are presented as the mean fluorescence intensity normalized to the A_{600nm} (relative fluorescence units). Error bars represent standard errors (SE) from three independent assays.

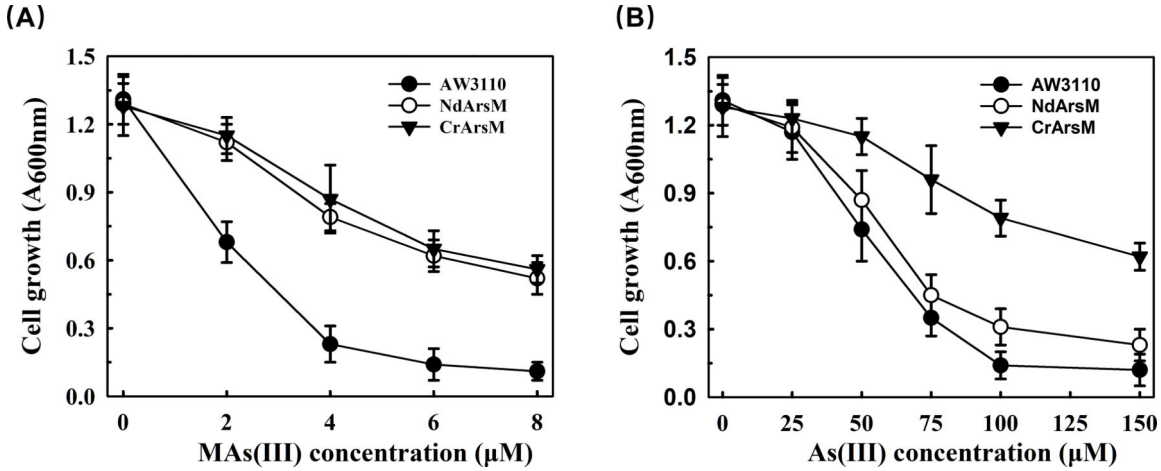

Fig. 5. Resistance to MAs(III)(A) or As(III)(B) conferred by expression of *arsM* genes.

E. coli strain AW3110 bearing plasmids pET29a-*NdarsM* (*arsM* gene from *Noviherbaspirillum denitrificans*), pET29a-*CrarsM* (*arsM* gene from *Chlamydomonas reinhardtii*), or vector plasmid pET29a were grown in M9 medium with 0.3 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 24 h at 37°C with shaking at 200 rpm, cell growth was estimated from the A_{600nm}. Error bars represent standard errors (SE) from three independent assays.

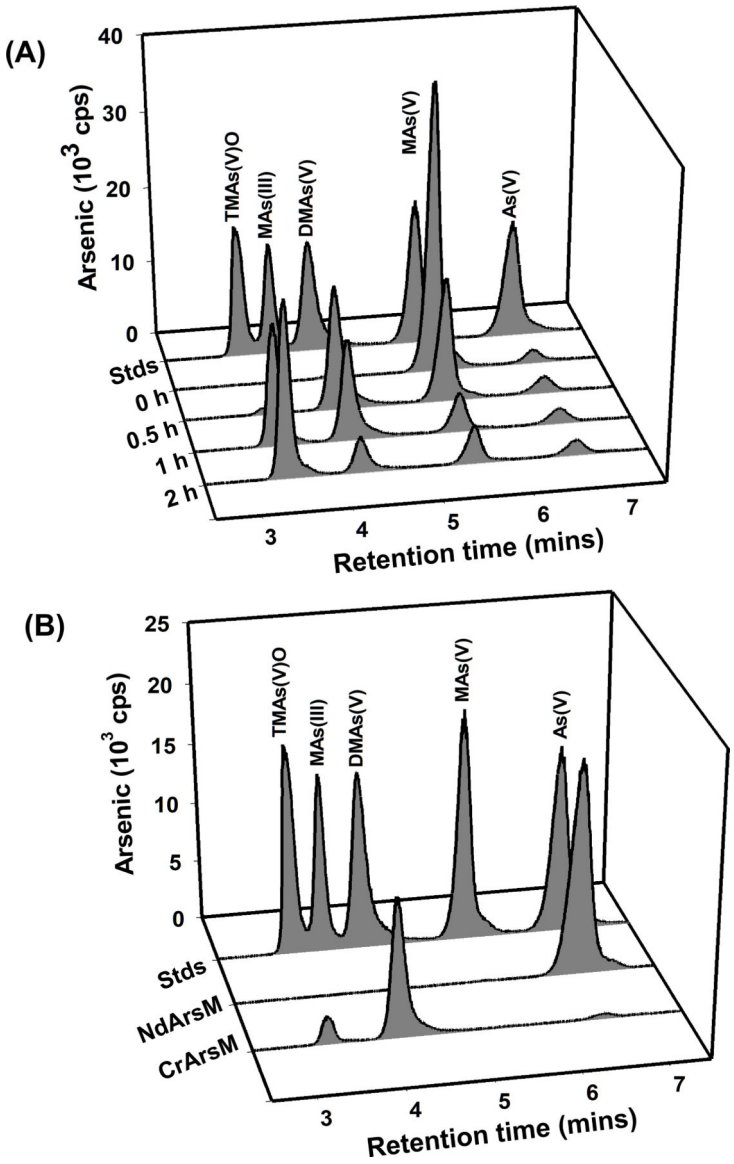

Fig. 6. Biomethylation of As(III) or MAs(III) by *E. coli* AW3110 expressing *NdarsM* or *CrarsM*.

Cell of *E. coli* AW3110 with plasmid pET29a-*NdarsM* (*arsM* gene from *Noviherbaspirillum denitrificans*) or pET29a-*CrarsM* (*arsM* gene from *Chlamydomonas reinhardtii*) was grown in M9 medium containing 4 μM MAs(III) (A) or 4 μM As(III) (B) plus 0.3 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 2 h. Samples were treated with 6% (v/v) H_2O_2, final concentration. Arsenic species in the medium were determined by HPLC-ICP-MS.

Table 1.

Methylation of MAs(III) or As(III) by NdArsM in *vitro*.

The initial concentration of MAs(III) or As(III) was 10 μM. Arsenic species were determined by HPLC-ICP-MS after samples were treated with 6% (v/v) H_2O_2. ND, not detected. Numbers in parentheses are the percentage of added arsenic. Data are the mean ± SE (n = 3).

Substrate(10 μM)	Time (h)	Products found in the reaction buffer (μM)			
		MAs(V)	DMAs(V)	TMAs(V)O	As(V)
MAs(III)	0	9.85 ± 0.13 (98.5%±1.3%)	ND	ND	0.05 ± 0.00 (0.5% ± 0.0%)
	0.5	5.60 ± 0.13 (56.0%±1.3%)	3.22 ± 0.17 (32.2% ± 1.7%)	0.62 ± 0.00 (6.2% ± 0.0%)	0.05 ± 0.00 (0.5% ± 0.0%)
	1.0	3.16 ± 0.09 (31.6%±0.9%)	4.93 ± 0.16 (49.3% ± 1.6%)	1.24 ± 0.06 (12.4% ± 0.6%)	0.05 ± 0.00 (0.5% ± 0.0%)
	1.5	1.6 ± 0.06 (16%±0.6%)	5.98 ± 0.14 (59.8% ± 1.4%)	1.99 ± 0.13 (19.9% ± 1.3%)	0.05 ± 0.00 (0.5% ± 0.0%)
	2.0	0.02 ± 0.00 (0.2% ± 0.0%)	6.5 ± 0.18 (65.0% ± 1.8%)	2.84 ± 0.15 (28.4% ± 1.5%)	0.05 ± 0.00 (0.5% ± 0.0%)
As(III)	0	ND	ND	ND	9.88 ± 0.2 (98.8% ± 2.0%)
	4	ND	ND	ND	9.87 ± 0.25 (98.7% ± 2.5%)