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Selective methylation by an ArsM S-adenosylmethionine methyltransferase from *Burkholderia gladioli* GSRB05 enhances antibiotic production

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Abbreviations: As(III), arsenite; As(V), arsenate; MAs(III), methylarsenite; MAs(V), methylarsenate; DMAs(V), dimethylarsenate; DMAs(III), dimethylarsenite; TMAs(V)O, trimethylarsine oxide; arsinothricin, AST; AST-OH, pentavalent hydroxyarsinothricin; R-AST-OH, trivalent hydroxyarsinothricin; AsS, arsenosugar; SAM, *S*-adenosylmethionine; HPLC, high pressure liquid chromatography; ICP-MS, inductively coupled plasma mass spectroscopy.

Synopsis: Here we show how the soil bacterium *Burkholderia gladioli* can enhance production of the novel arsenic-containing antibiotic arsinothricin.

Key words: ArsM, SAM methyltransferase, arsinothricin, hydroxyarsinothricin, arsenic-containing antibiotic

Running Title: An ArsM specific for antibiotic biosynthesis

ABSTRACT

Arsenic methylation contributes to the formation and diversity of environmental organoarsenicals, an important process in the arsenic biogeochemical cycle. The *arsM* gene encoding an arsenite (As(III)) *S*-adenosylmethionine (SAM) methyltransferase is widely distributed in members of every kingdom. A number of ArsM enzymes have been shown to have different patterns of methylation. When incubated with inorganic As(III) *Burkholderia gladioli* GSRB05 has been shown to synthesize the organoarsenical antibiotic arsinothricin (AST), but does not produce either methylarsenate (MAs(V)) or dimethylarsenate (DMAs(V)). Here we show that cells of *B. gladioli* GSRB05 synthesize DMAs(V) when cultured with either MAs(III) or MAs(V). Heterologous expression of the *BgarsM* gene in *Escherichia coli* conferred resistance to MAs(III) but not As(III). The cells methylate MAs(III) and the AST precursor, reduced trivalent hydroxyarsinothricin (R-AST-OH) but do not methylate inorganic As(III). Similar results were obtained with purified BgArsM. Compared with ArsM orthologs, BgArsM has an additional 37 amino acid residues in a linker region between domains. Deletion of the additional 37 residues restored As(III) methylation activity. Cells of *E. coli* co-expressing the *BgarsL* gene encoding the noncanonical radical SAM enzyme that catalyzes synthesis of R-AST-OH together with the *BgarsM* gene produce much more of the antibiotic AST compared with *E. coli* cells co-expressing *BgarsL* together with the *CrarsM* gene from *Chlamydomonas reinhardtii,* which lacks the sequence for additional 37 residues. We propose that the presence of the insertion reduces the fitness of *B. gladioli* because it cannot detoxify inorganic arsenic but concomitantly confers an evolutionary advantage by increasing the ability to produce AST.

INTRODUCTION

Arsenic is a naturally occurring metalloid that is widely distributed throughout the environment in air, water and soil.[1] Inorganic arsenic in the form of both arsenite (As(III)) and arsenate (As(V)) is the most common environmental contaminant. It can form a wide variety of organoarsenicals with As-C bonds,[2] such as methylarsenite (MAs(III)), arsenobetaine, arsenosugars (AsS) and the newly identified organoarsenical antibiotic arsinothricin (AST).[3] Bioavailability and toxicological properties of arsenicals are highly dependent on their chemical forms and oxidation states. Microbial arsenic methylation constitutes a critical component of arsenic biogeochemical cycles.[4] As(III) can be methylated sequentially to mono-, di- and trimethylated species, catalyzed by As(III) SAM methyltransferases (ArsM in microbes and AS3MT in animals).[5, 6] The trivalent methylated products are readily oxidized in air to much less toxic pentavalent methylarsenate (MAs(V), dimethylarsenate (DMAs(V)) and trimethylarsine oxide (TMAsO(V)), which led to the concept that arsenic methylation is a detoxification process.[7]

In addition, microbially-mediated arsenic methylation is a significant source of more complex organoarsenicals such as AsS and arsinothricin [2-amino-4-(hydroxymethylarsinoyl) butanoate] or AST).[8] In the *Synechocystis* sp. PCC 6803 *arsMS* operon there are two steps in AsS biosynthesis.[9, 10] The first step is transfer of methyl groups from SAM to As(III) by ArsM, producing DMAs(III). The second step is addition of the deoxyribose moiety of SAM to DMAs(III) by the ArsS radical SAM enzyme, forming 5'-deoxy-5'-dimethylarsinoyl-adenosine, the precursor of many arsenosugars and arsenolipids.[9] In the *Burkholderia gladioli* GSRB05 *arsQML* operon, the noncanonical radical SAM enzyme ArsL cleaves the C-C bond of SAM, forming a 3-amino-3-carboxypropyl (ACP) radical. This reacts with As(III) to form a C-As bond, producing the trivalent form of the AST precursor hydroxyarsinothricin [2-amino-4-(dihydroxyarsinoyl) butanoate] (R-AST-OH). ArsM transfers a methyl group from SAM to R-AST-OH, forming the

trivalent form of the antibiotic AST.[11] These two examples illustrate the capability of ArsM enzymes to participate in production of a wide variety of organoarsenicals.

Most ArsMs have four conserved cysteine residues that appear to direct substrate specificity. All four cysteines are required for As(III) methylation, but only the last two cysteines are required for MAs(III) methylation.[12] Four-cysteine ArsMs can be operationally classified into two subgroups based on methylation activity.[13] Enzymes in Group 1 such as mammalian AS3MT exhibit relatively low arsenic methylation activity and rarely produce trimethylated arsenic. Enzymes in Group 2 rapidly methylate arsenic such as ArsM from *Rhodopseudomonas palustris* and are capable of producing volatile trivalent trimethylated TMAs(III).[6] The crystal structure of CmArsM, from the acidothermophilic red alga *Cyanidioschyzon merolae* sp. 5508, shows an N-terminal domain with the SAM binding site, a central domain with the binding site for As(III) or MAs(III) and a C-terminal domain of unknown function.[14] However, some ArsMs have fewer than four cysteines or have a different domain structure. For example, ArsM from the fungus *Aspergillus fumigatus* has three of the four conserved cysteines and methylates MAs(III) but not As(III).[15] ArsM from *Bacillus* sp. CX-1 has three conserved cysteines but methylates As(III) with only two.[16] An atypical ArsM from *Thermus thermophilus* HB27 has only a single conserved cysteine and yet is still capable of As(III) methylation.[17] ArsM from *Noviherbaspirillum denitrificans* HC18 has all four conserved cysteines but lacks the C-terminal domain. It retains MAs(III) methylation activity but does not methylate As(III).[18] These ArsMs may be an evolutionary step on the pathway to the canonical four-cysteine ArsM or may be derivatives that evolved differently in response to changing environmental conditions. A more complete molecular genetic and biochemical analysis of different ArsMs is required to fill out the evolutionary history.

This study focuses on one such noncanonical ArsM, BgArsM from *B. gladioli* GSRB05, which functions with BgArsL in AST biosynthesis. No MAs(V) or DMAs(V) was detected when *B.*

gladioli was cultured with As(III), suggesting that BgArsM may not be capable of methylating As(III).[11] Here we show that BgArsM can methylate organoarsenicals, such as MAs(III) and R-AST-OH to DMAs and AST, respectively. Co-expression of BgArsM and BgArsL in *E. coli* resulted in AST production. Less AST was produced when CrArsM (AFS88933) from *C. reinhardtii*[19] was co-expressed with BgArsL, even though CrArsM is a highly efficient As(III) methylator. Deletion of the additional 37 residues from BgArsM restored As(III) methylation activity. We propose that BgArsM acquired the additional residues to prevent As(III) from being methylated to MAs(III), increasing methylation of R-AST-OH produced by BgArsL. This would channel As(III) into more efficient AST production at the expense of arsenic detoxification. These results will be valuable for understanding the regulation of biosynthesis of this novel arsenical antibiotic.

MATERIALS AND METHODS

Chemicals

Unless otherwise indicated, chemicals were purchased from Sigma-Aldrich (St. Louis, MO). MAs(V) was obtained from Chem Service (West Chester, PA). MAs(V) and AST-OH were reduced as described.[20] Briefly, 0.2 mM arsenicals were mixed with 27 mM $Na_2S_2O_3$, 66 mM $Na_2S_2O_5$, and 82 mM H_2SO_4, following which the pH was adjusted to 6.0 with NaOH. Biosynthetic generated L-AST was purified as described.[11] D, L-AST-OH was chemically synthesized.[21]

Strains, medium and growth conditions

E. coli Stellar™ (Clontech Laboratories, Mountain View, CA) (*F⁻, endA1, supE44, thi-1, recA1, relA1, gyrA96 phoA, Φ80d lacZΔ M15, Δ(lacZYA-argF)U169*, *Δ(mrrhsdRMS-mcrBC), ΔmcrA, λ–*) was used for plasmid DNA construction and replication. *E. coli* AW3110(DE3) (*Δars*::cam

123 *F−IN(rrn-rrnE)*,[22] which is hypersensitive to As(III), was used for complementation studies. *E.*
124 *coli* BL21(DE3) (Novagen, Madison, WI) was used for protein expression. *B. gladioli* GSRB05,
125 which was isolated from rice rhizosphere [8], and *E. coli* cultures were grown aerobically with
126 shaking at either 30 or 37°C in either LB, M9 [23] or R2A medium,[24] as noted, supplemented with
127 125 μg/mL ampicillin, 50 μg/mL kanamycin or 34 μg/mL chloramphenicol, as required. Bacterial
128 growth was monitored from the absorbance at 600 nm (A_{600nm}).
129
130 **Plasmid construction**
131 A *BgarsM* gene encoding BgArsM (accession number WP_219608244) from the *B. gladioli*
132 GSRB05 genome (NZ_JAGSIB010000059.1) was chemically synthesized with 5' *Nco*I and 3'
133 *Xho*I sites and with codon optimization for expression in *E. coli* and subcloned into the *EcoR*V
134 site of vector plasmid pUC57-Kan (GenScript, NJ, USA). The synthetic *BgarsM* gene was
135 cloned as an *Nco*I/*Xho*I double-digested fragment from pUC57-Kan-*BgarsM* into expression
136 vector pET28a (Novagen, Madison, WI), generating plasmid pET28a-*BgarsM*. *CrarsM* gene in
137 pET28a(+) was used as a positive control.[19] To delete extra 37 amino acids, two *Bgl*II restriction
138 sites were introduced in *BgarsM* by site-directed mutagenesis using a QuikChange II Site-
139 Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA). *BgarsM* mutant plasmid with
140 two *Bgl*II sites was digested by *Bgl*II enzyme to remove the extra amino acids and self-ligated to
141 generate plasmid pET28a-$BgarsM_{\Delta 37}$. Conserved cysteine residues in BgArsM were changed to
142 serine residues using the same mutagenesis protocol. To show the evolutionary advantage of
143 BgArsM in AST production, *BgarsL* was co-expressed with either *BgarsM* or *CrarsM* in
144 expression vector pETDuet-1 (Millipore Sigma, Burlington, MA), generating plasmid pETDuet-
145 *BgarsL-BgarsM* and pETDuet-*BgarsL-CrarsM,* respectively. Plasmids used in this study are
146 described in Table S1 in the supplemental material. Primers used for plasmid constructions and

site mutagenesis are listed in Table S2. Each construct was confirmed by DNA sequencing (Sequetech, Mountain View, CA).

Phylogenetic analysis

Multiple alignment of ArsM homologous sequence was calculated with CLUSTAL W.[25] ArsM sequences with conserved cysteines were selected for phylogenetic analysis. Acquisition of sequences was performed by searching a list of reference organisms or from the National Center for Biotechnology (NCBI) protein database by BLASTP search.[26] Phylogenetic analysis was performed to infer the evolutionary relationship among the ArsMs of various organisms. The phylogenetic tree was constructed using the Neighbor-Joining method using MEGA 6.0.1.[27] The statistical significance of the branch pattern was estimated by conducting 1000 bootstrap replicates.

Assay of arsenicals biotransformation

Cells of *B. gladioli* GSRB05 and *E. coli* AW3110 with various *arsM* constructs were cultured aerobically with shaking in LB medium overnight at 30 °C, with 0.3 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) as an inducer, as required. The cells were washed once and suspended in R2A medium[24] without glucose at a cell density of A_{600nm} = 3.0. Arsenicals were then added at 5 μM, final concentration, to the cell suspensions, which were incubated at 30 °C with shaking for 4 h. As noted, soluble arsenicals were treated with 6% (v/v) H_2O_2 and heated at 80 °C for 5 min to oxidize all arsenic species. Samples were speciated by high pressure liquid chromatography (HPLC) coupled to inductively coupled plasma mass spectroscopy (ICP-MS) using a BioBasic-18 5 μm C18 300 Å reverse-phase column (250 mm × 4.6 mm; Thermo Fisher Scientific, Waltham, MA) eluted isocratically with a mobile phase consisting of 3 mM malonic acid and 5% methanol (vol/vol), pH 5.6 (adjusted by tetrabutylammonium hydroxide), with a flow

rate of 1 mL min^{-1} at 25 °C. To trap volatile arsenicals, 2 cm nitrocellulose membrane filters were put in vial caps and impregnated with 0.15 mL of 6% H_2O_2.[6] The filters were digested with 0.2 mL of 70% HNO_3 at 70 °C for 20 min. The digestion solutions were diluted 25-fold and speciated by HPLC-ICP-MS with anion exchange column (250 mm × 4.1 mm, 10 µm, 300 Å, PRP-X100, Hamilton Company, Reno, NV) eluted with a step gradient composed of 9 mL of mobile phase A (20 mM ammonium bicarbonate, pH 8.5) and 18 mL of mobile phase B (20 mM ammonium sulfate, pH 7.0) at a flow rate of 1.5 ml/min. Some arsenic remained bound to cellular constituents and was not recovered.

Metalloid resistance assays

For metalloid resistance assays, competent cells of AW3110 (DE3) were transformed with constructs bearing plasmid pET28a-*BgarsM*, pET28a-$BgarsM_{\Delta 37}$, or pET28a-*CrarsM*. Cells were grown overnight with shaking at 37 °C in LB medium with 50 µg/mL Kanamycin. Overnight cultures were diluted 100-fold in M9 medium containing various concentrations of As(III) or MAs(III) plus 0.3 mM IPTG and incubated at 37 °C with shaking for an additional 24 h. Growth was estimated from the absorbance at 600 nm.

BgArsM purification

E. coli BL21(DE3) cells (Thermo Fisher Scientific) bearing plasmid pET28a-*BgarsM* and its derivates were grown in LB medium containing 50 µg/mL Kanamycin with shaking at 37 °C. Cells at an A_{600nm} of 0.6 were induced by 0.3 mM IPTG and further cultured for 4 h. The cells were harvested and suspended in 5 mL per gram of wet cells in buffer A (50 mM 4-morpholinepropanesulfonic acid (MOPS), 20 mM imidazole, 0.5 M NaCl, 10 mM 2-mercaptoethanol and 20% glycerol (vol./vol.), pH 7.5). The cells were broken by a single passage through a French pressure cell at 20,000 psi and immediately treated with the protease

197 inhibitor diisopropyl fluorophosphate (2.5 µL per gram wet cell). Membranes and unbroken cells
198 were removed by centrifugation at 150,000g for 1 h, and the supernatant solution was loaded
199 onto a Ni^{2+}-nitrilotriacetic acid column (Qiagen, Valencia, CA) at a flow rate of 0.5 mL min^{-1}. The
200 column was washed with more than 25 column volumes of buffer A. BgArsM was eluted with
201 buffer A containing 0.2 M imidazole, and the purity was analyzed by sodium dodecyl sulfate-
202 polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentrations were estimated from
203 A_{280nm} (ε = 22 940 M^{-1} cm^{-1} for BgArsM). BgArsM-containing fractions were divided into portions,
204 rapidly frozen and stored at -80 °C until use.
205
206 **Organoarsenicals methylation by purified BgArsM**
207 Methylation activity of purified BgArsM and its derivates were assayed at 37 °C in buffer
208 consisting of 50 mM MOPS, pH 7.5, containing 0.3 M NaCl, 8 mM glutathione (GSH) and 1 mM
209 SAM. As(III), MAs(III) or R-AST-OH (10 µM) was incubated at 37 °C in the presence or absence
210 of 3 µM BgArsM. Reactions were collected after 6 h, and protein was removed by centrifugation
211 using a 3 kDa cutoff Amicon ultrafilter (MilliporeSigma, Burlington, MA). The filtrate was
212 speciated by HPLC-ICP-MS. Where noted, H_2O_2 was not added to allow for determination of
213 trivalent arsenicals.
214
215 **Homology modeling of BgArsM**
216 The BgArsM homology model was built using SWISS-MODEL online server Kiefer et al., 2009).
217 The crystal structure of CmArsM (PDB ID: 4FR0) was used as template.[14] The QMEAN and
218 GMQE scores of the model are −4.46 and 0.45, respectively, indicating a satisfactory quality of
219 the model. The modelled structure was analyzed and figures were generated using PyMOL
220 (PyMOL Molecular Graphics System, Version 1.3, Schrodinger LLC (http://www.pymol.org/) and
221 UCSF Chimera,[28]

RESULTS AND DISCUSSION

Arsenic biotransformations

The soil bacterium *B. gladioli* GSRB05 can transform As(III) into the natural product AST, which has been demonstrated to be a broad-spectrum antibiotic.[3] In the AST biosynthetic gene cluster, of *B. gladioli* GSRB05 there is an *arsM* gene adjacent to *arsL*.[11] In this study the ability of *B. gladioli* GSRB05 to methylate inorganic and organic arsenicals was examined (Fig. 1). AST-OH and AST were detected when *B. gladioli* GSRB05 was cultured with either As(III) or As(V) (Fig. 1A). Since As(V) is assumed to be reduced to As(III) by *B. gladioli* GSRB05 ArsC[29] (MBW5287232), both can be transformed to AST. DMAs(V) was not converted to AST, but small amounts of thiolated DMAs(V) species were detected (Fig. 1A). Over a 25 h time period of MAs(III) exposure, DMAs(V), AST-OH, AST and a small amount of As(V) were all detected, with AST as the predominant product (Fig. 1B). These complex metabolic biotransformations reflect the presence of *arsM*, *arsL*, *arsC* genes and an *arsI* gene encoding an MAs(III)-demethylating ArsI enzyme (MBW5287228) in the *B. gladioli* genome. ArsI can demethylate MAs(III) to As(III)[30], which can be oxidized to As(V) by the AioA (WP_219608272.1) and AoiB (WP_219608248) complex[31] or converted to AST-OH and AST by ArsL and ArsM. MAs(III) might also be able to be directly converted to AST by BgArsL. Biotransformation of MAs(V) was similar to that of MAs(III) (Fig. 1C). Presumably MAs(V) can be reduced to MAs(III) by a mechanism similar to that previously identified in *Burkholderia* sp. MR1,[32] *Shewanella putrefaciens* 200,[33] and *Sinorhizobium meliloti* RM1021.[34] The MAs(III) concentration increased with incubation time up to 20 h, after which it decreased, while DMAs(V) and AST continued to increase. After 60 h of incubation with MAs(V), AST and thiolated DMMTAs(V) were the predominant products. *B. gladioli* GSRB05 can also directly methylate trivalent R-AST-OH to

AST (Fig. 1D). These results indicate that cells of *B. gladioli* GSRB05 do not methylate As(III) to MAs(III) but can methylate MAs(III) to DMAs(V) and R-AST-OH to AST.

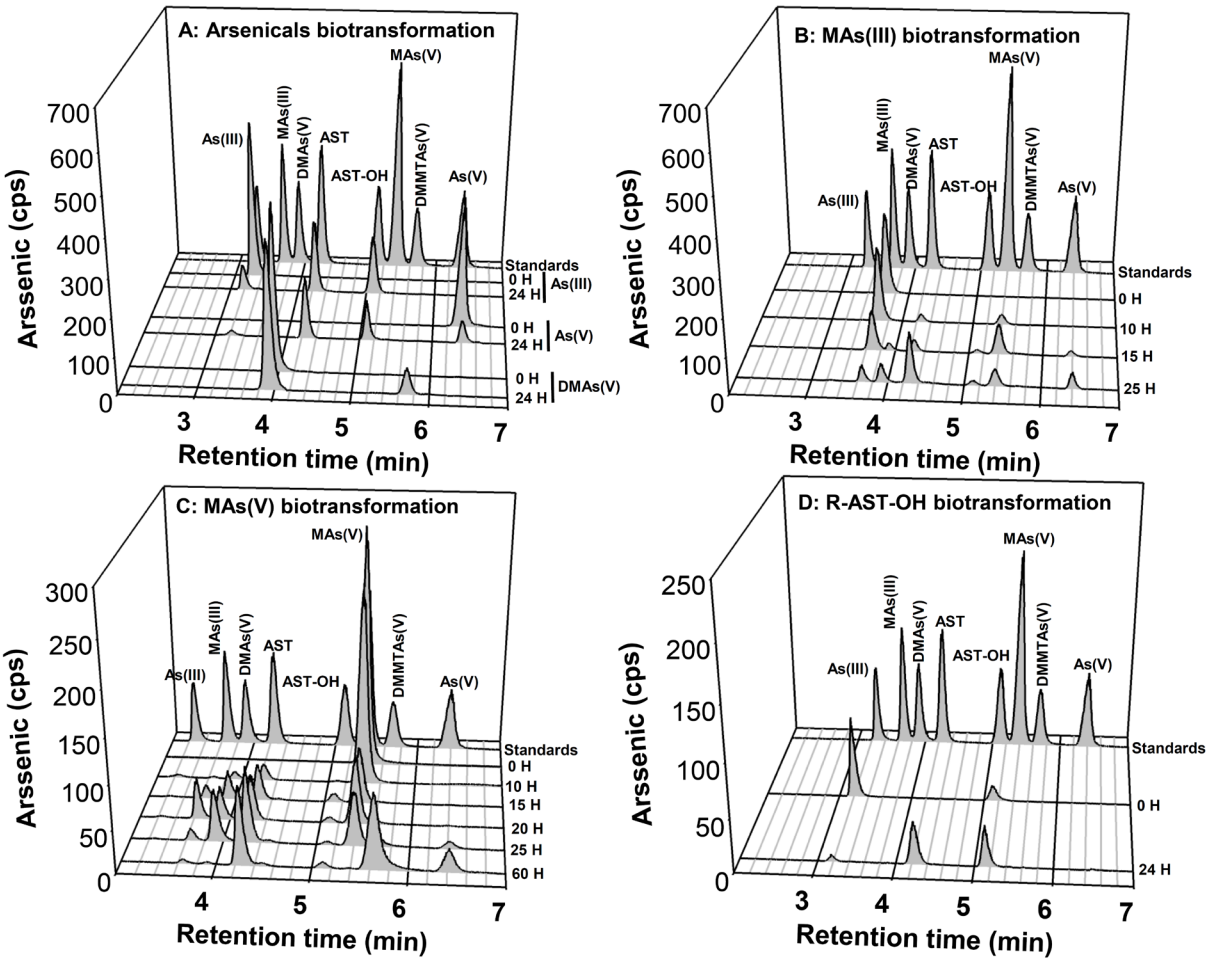


Figure 1. Arsenic biotransformations by cells of *B. gladioli* GSRB05. (A) As(III), As(V) and DMAs(V) biotransformations. (B) Time course of MAs(III) transformation. (C and D) Time course of MAs(V) and R-AST-OH biotransformations. Cells of *B. gladioli* GSRB05 were cultured overnight in LB medium, washed and suspended at a density of A_{600} of 3.0 in R2A medium, then incubated at 30 °C with the indicated arsenicals, each at 5 μM final concentration. After the indicated times, samples were speciated by HPLC using a C18 reverse phase column, and the amount of arsenic was estimated by ICP-MS.

BgArsM from *B. gladioli* GSRB05 methylates trivalent R-AST-OH but not As(III)

BgArsM has been shown to methylate trivalent R-AST-OH to AST.[11] Like most bacterial, algal and mammalian ArsM orthologs, BgArsM has four conserved cysteine residues at positions 30, 54, 181 and 233. Multiple sequence alignment with orthologs shows that BgArsM has an additional 37 residues in a linker between its N-terminal and central domains (Fig. S1). BgArsM (WP_219608244) shows 30% identity and 38% similarity with CmArsM from the thermophilic eukaryotic alga *Cyanidioschyzon* sp. 5508 (ACN39191.1), 29% identity and 37% similarity to CrArsM (AFS88933) from the alga *C. reinhardtii* and 37% identity and 38% similarity to RpArsM from the photosynthetic bacterium *Rhodopseudomonas palustris* (WP_011159102), respectively. To examine the evolutionary relation of BgArsM with other members of the ArsM family from prokaryotes and eukaryotes organisms, a phylogenetic analysis of BgArsM sequences was conducted (Fig. 2). ArsM orthologs from bacteria, animals, algae, fungi and archaea cluster in individual subgroups. Bacterial ArsMs fall into three subgroups, one of which includes BgArsM, which is required for AST biosynthesis. ArsMs involved in arsenosugar biosynthesis cluster in a separate bacterial subgroup. The grouping suggests that BgArsM has different methylation properties from other bacterial ArsMs.

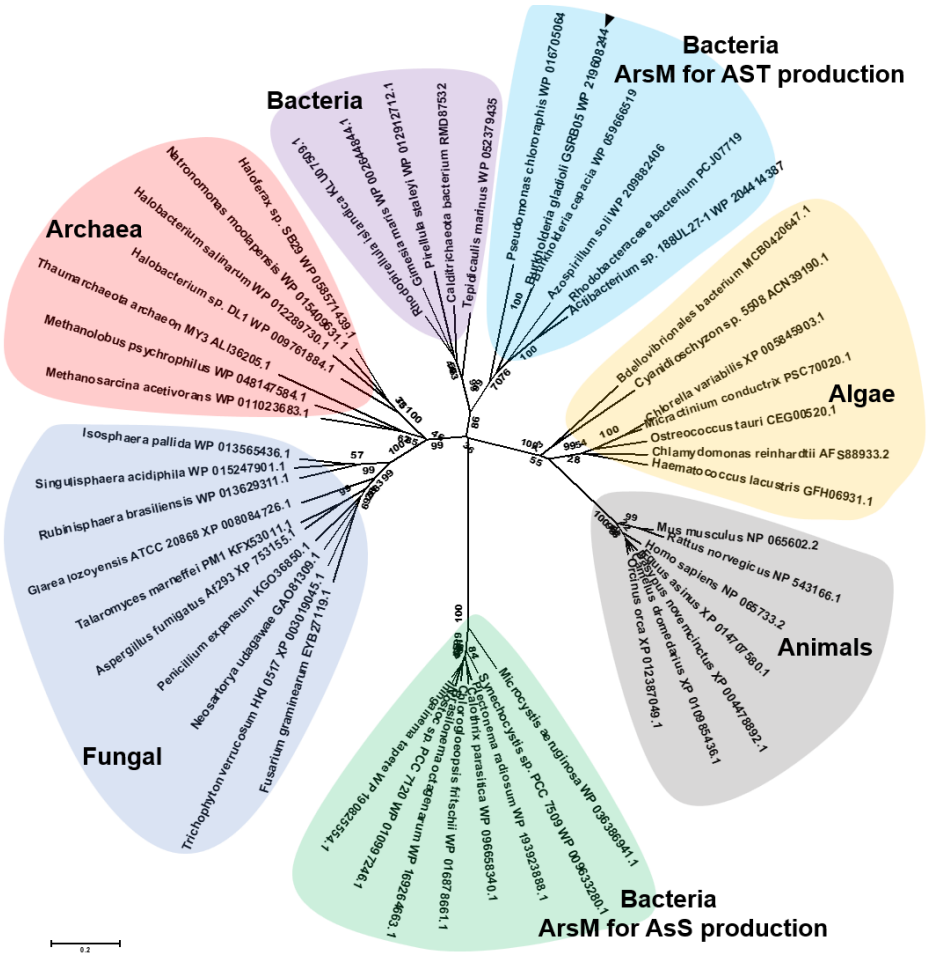


Figure 2. A neighbor-joining phylogenetic tree showing the evolutionary relationship of BgArsM (black triangle). with arsenic methyltransferase proteins from members of other kingdoms.

To examine its methylation activity, The *BgarsM* gene was chemically synthesized with 5'-*Nde*I and 3'-*Xho*I sites at each end with codon optimization for expression in *E. coli* and subcloned into plasmid pET28a(+) (GenScript, NJ, USA). The *C. reinhardtii CrarsM* gene in pET28a(+) was used as a positive control. ArsMs were expressed in *E. coli* BL21(DE3), and the products of methylation were analyzed (Table 1). After 4 h of incubation, the reactions were terminated with H_2O_2, which oxidizes and solubilizes the products. Arsenic in the supernatant solution was speciated by HPLC-ICP-MS. When cells were incubated with 5 μM As(III), cells with pET28a-*BgarsM* showed poor As(III) methylation, with only 1.0% DMAs(V) and no volatile TMAsO(V)

detected. In contrast, cells with pET28a-*CrarsM* produced 80.4% DMAs(V) and 4.6% TMAsO(V). Both ArsMs methylated MAs(III) with high efficiency, producing 63.0% DMAs(V) and 24.6% TMAsO(V) by BgArsM and 60.2% DMAs(V) and 29.0%TMAsO(V) by CrArsM. These results demonstrate that BgArsM methylates MAs(III) but not As(III), consistent with the lack of methylated arsenical production by *B. gladioli* GSRB05 when treated with As(III) (Figure 1).

Table 1. Methylation of As(III) or MAs(III) by BgArsM or CrArsM expressed in *E. coli*

		Products found in culture medium (μM)[a]			
Substrate (5 μM)	ArsM	MAs(V)	DMAs(V)	TMAsO(V)	As(V)
As(III)	Vector	ND[b]	ND	ND	4.85±0.16 (97.0%±3.2%)
	CrArsM	0.14±0.03 (2.8%±0.6%)[c]	4.02±0.17 (80.4%±3.4%)	0.23±0.04 (4.6%±0.8%)	0.21±0.03 (4.2%±0.6%)
	BgArsM	ND	0.05±0.00 (1.00%±0.0%)	ND	4.81±0.18 (96.2%±3.6%)
MAs(III)	Vector	4.82±0.12 (96.4%±2.4%)	ND	ND	ND
	CrArsM	0.18±0.05 (3.6%±1.0%)	3.01±0.13 (60.2%±2.6%)	1.45±0.09 (29.0%±1.8%)	ND
	BgArsM	0.21±0.04 (4.2%±0.8%)	3.15±0.16 (63.0%±3.2%)	1.23±0.07 (24.6%±1.4%)	ND

a. Methylation activity was assayed in *E. coli* cells expressing *BgarsM* or *CrarsM*, as described in Materials and Methods. Cells were incubated with As(III) or MAs(III) at 5 μM, final concentration. All samples were treated with 6% (v/v) H_2O_2, final concentration, and separated by HPLC using a C18 reverse phase column, and the amount of arsenic was estimated by ICP-MS. Data are the mean ± SE (n=3).

b. ND, not detected.

c. Numbers in parentheses are the percentage of added arsenic.

BgArsM was purified from *E. coli* and assayed for methylation activity. Purified BgArsM methylated R-AST-OH to AST (Fig. 3A). To examine the role of conserved cysteines in organoarsenical methylation, each of the four cysteines was altered individually to serine residues. Purified derivative C30S and C54S methylated MAs(III) similarly to wild type BgArsM (Fig. 3B), but neither the C181S nor C233S enzymes exhibited MAs(III) methylation activity. These results demonstrate that BgArsM is selective for trivalent organoarsenicals and does not methylate As(III).

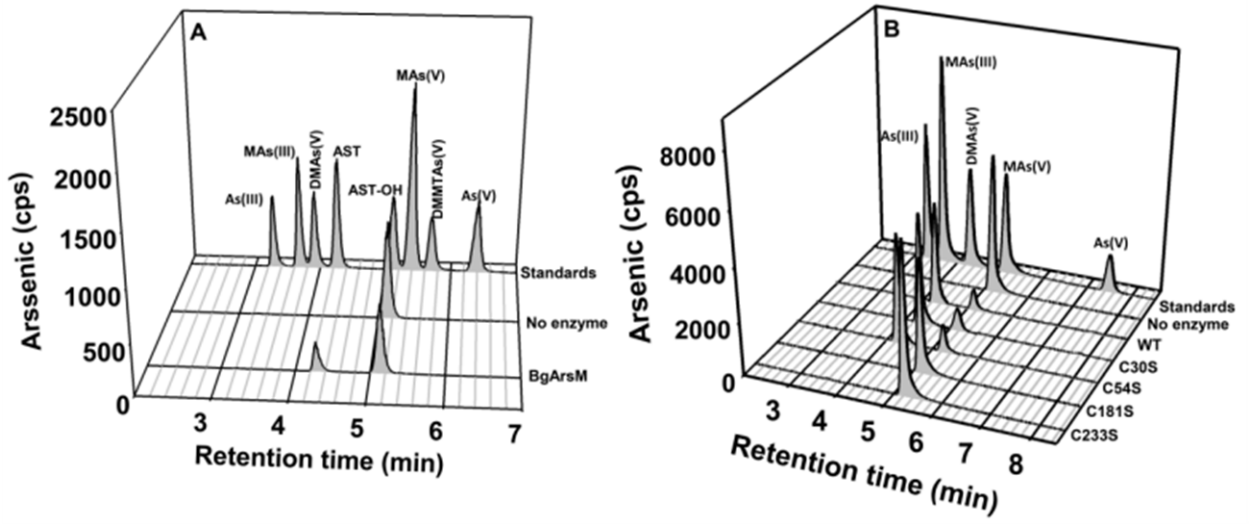
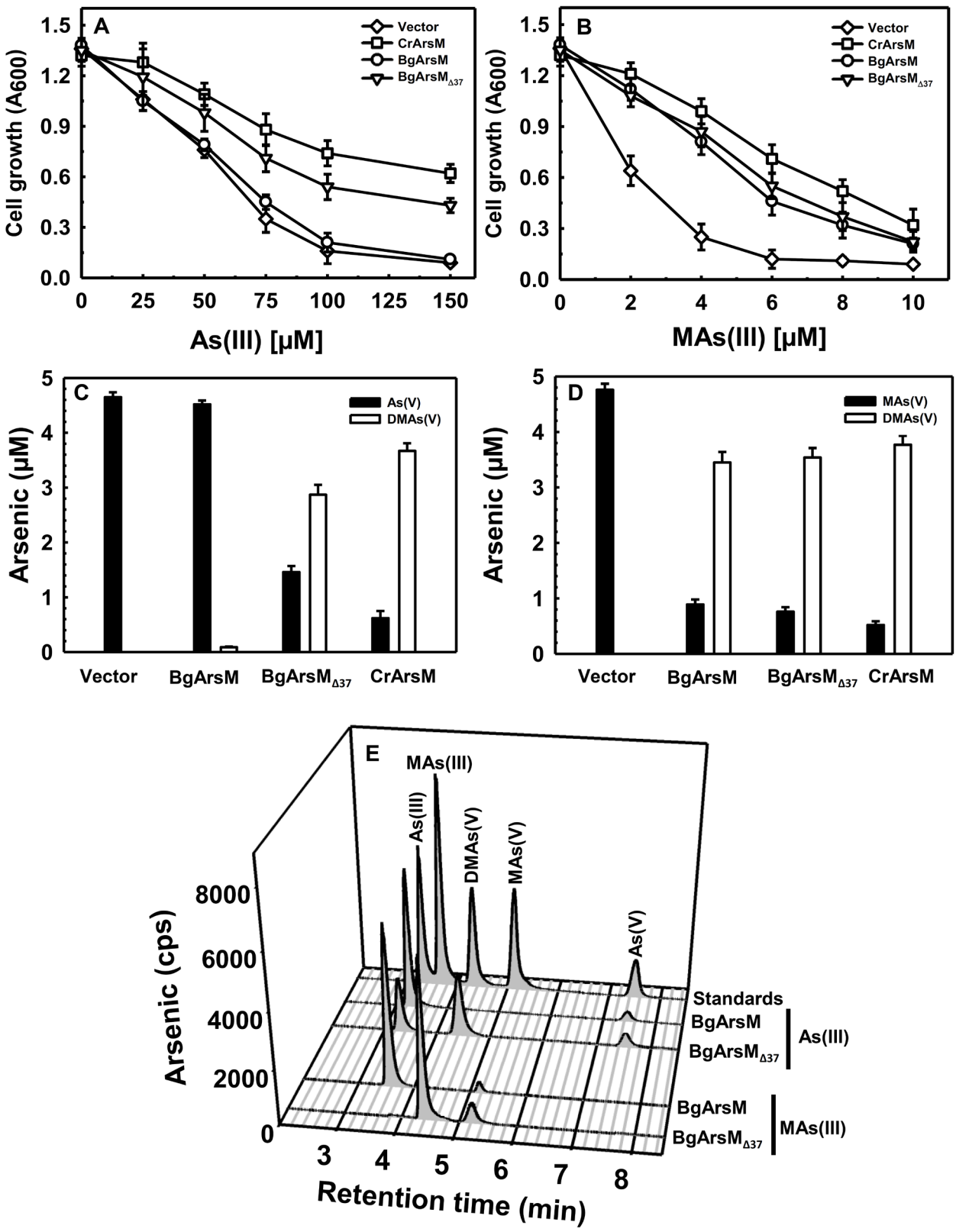


Figure 3. Methylation of R-AST-OH or MAs(III) by purified BgArsM. (A) Purified BgArsM methylates R-AST-OH. (B) Effect of alteration of conserved cysteine residues in BgArsM on

MAs(III) methylation. BgArsM or variants were purified and assayed for methylation of R-AST-OH or MAs(III), as described in Materials and Methods. The reaction mixture (1 mL) containing 3 μM purified BgArsM or variants, 1 mM SAM, 8 mM GSH and 10 μM of either R-AST-OH or MAs(III) was incubated at 37 °C for 6 h. All reactions were terminated by addition of 6% (v/v) H_2O_2, final concentration. Arsenicals were separated by HPLC using a C18 reverse phase column, and the amount of arsenic was estimated by ICP-MS.

Deletion of the additional amino acids restores As(III) methylation activity

Compared to other ArsM orthologs, BgArsM has 37 additional residues between the N-terminal SAM binding domain and the central As(III) binding domain (Fig. S1). We considered the possibility that the insertion was responsible for inability to methylate As(III). To examine whether these extra amino acids affect As(III) methylation, a *BgarsM*$_{\Delta 37}$ gene was constructed in which the DNA sequence encoding the 37 amino acid residues was deleted. The mutant gene was expressed in the *E. coli* As(III)-hypersensitive strain AW3110 (*Δars*).[22] Expression of the wild type *BgarsM* gene did not complement the As(III) sensitive phenotype (Fig. 4A) but did confer resistance to MAs(III) (Fig. 4B). Expression of CrArsM, which lacks the sequence corresponding to the additional residues, conferred resistance to both MAs(III) and As(III). In contrast, cells of *E. coli* AW3110 (*Δars*) expressing BgArsM$_{\Delta 37}$ showed increased As(III) resistance (Fig. 4A) but not MAs(III) resistance (Fig. 4B). Cells expressing BgArsM$_{\Delta 37}$ methylated both As(III) and MAs(III) (Fig. 4C and D) and could methylate As(III) to DMAs(V), while MAs(III) methylation activity was not changed. Purified BgArsM methylated MAs(III) but not As(III). In contrast, purified BgArsM$_{\Delta 37}$ methylated both As(III) to MAs(III), with DMAs(V) as the main product (Fig. 4E). Clearly, the additional 37 residues in some way prevent As(III) methylation.


A
Vector
CrArsM
BgArsM
BgArsM$_{\Delta37}$
Cell growth (A600)
As(III) [μM]
B
MAs(III) [μM]
C
As(V)
DMAs(V)
Arsenic (μM)
Vector
BgArsM
BgArsM$_{\Delta37}$
CrArsM
D
MAs(V)
DMAs(V)
E
MAs(III)
As(III)
DMAs(V)
MAs(V)
As(V)
Arsenic (cps)
Standards
BgArsM
BgArsM$_{\Delta37}$
As(III)
BgArsM
BgArsM$_{\Delta37}$
MAs(III)
Retention time (min)

336

Figure 4. Deletion of the additional 37 amino acid residues restores As(III) methylation activity. Resistance to As(III) (**A**) or MAs(III) (**B**) conferred by expression of the *BgarsM*$_{\Delta 37}$ gene. Cells of *E. coli* AW3110(DE3) bearing plasmids pET28a-*BgarsM*, pET28a-*BgarsM*$_{\Delta 37}$, pET28a-*CrarsM* or vector plasmid pET28a were grown in M9 medium with 0.3 mM 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. Biomethylation of As(III) or MAs(III) by BL21(DE3) expressing *BgarsM*, *BgarsM*$_{\Delta 37}$ or *CrarsM* genes was assayed. Cells of BL21(DE3) were grown in LB medium overnight, transferred to M9 medium containing 5 μM As(III) (**C**) or 5 μM MAs(III) (**D**) and incubated at 30°C with shaking for 6 h. Samples were treated with 6% (v/v) H_2O_2, final concentration. Arsenic species in the medium were determined by HPLC-ICP-MS. (**E**) Methylation of As(III) or MAs(III) by purified BgArsM or BgArsM$_{\Delta 37}$. Enzymatic activity of purified enzymes was assayed as described in Materials and Methods.

Homology modeling of BgArsM suggests that the additional 37 residues are in a regulatory site.

How do the additional 37 residues in BgArsM prevent methylation of inorganic As(III), while the deletion mutant regains that activity? From examination of the sequence from the 100 closest ArsM sequences, nothing remarkable about the sequence was noted (Fig. S2). No single residue was conserved in all 100 sequences, which were a mixture of polar and nonpolar residues with no obvious structural elements. This suggests that the 37 residues may form an unstructured loop. To predict how the additional residues might affect the structure and function of BgArsM, a homology structural model of BgArsM was constructed based on the crystal structure of CmArsM with bound SAM (PDB ID: 4FR0).[14] In CmArsM the structure can be considered of having two halves, with a cleft that is formed at their interface that ends at the As(III) binding site. Capping the cleft is a small loop (wheat) in the CmArsM structure (Fig. 5A).

The additional 37 residues in BgArsM are in this small loop, considerably increasing the size of the predicted loop in the homology structural model (Fig. 5B). We previously identified small molecule inhibitors of CmArsM and the human ortholog AS3MT.[35] One group of inhibitors prevent As(III) methylation but not MAs(III) methylation. From *in silico* docking analysis, those inhibitors appear to bind in the cleft between the two halves. We interpreted these results to suggest that the cleft is a regulatory region that opens and closes during catalysis, and that the inhibitors restrict the conformational change associated with As(III) methylation. It is not clear why they do not prevent methylation of MAs(III). It is not likely to be coincidence that the extra 37 residues appear to be located at the interface of the two halves of BgArsM. We predict that the larger "cap" that restricts conformational changes and prevents As(III) methylation in a manner similar to the small molecule inhibitors.

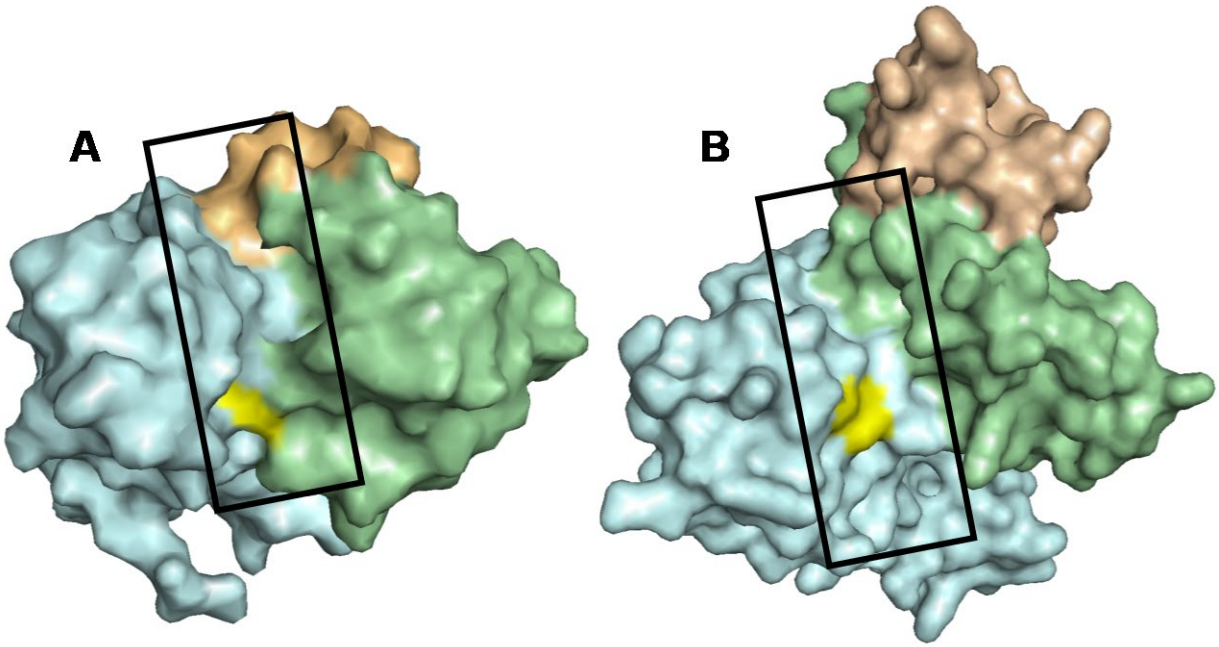
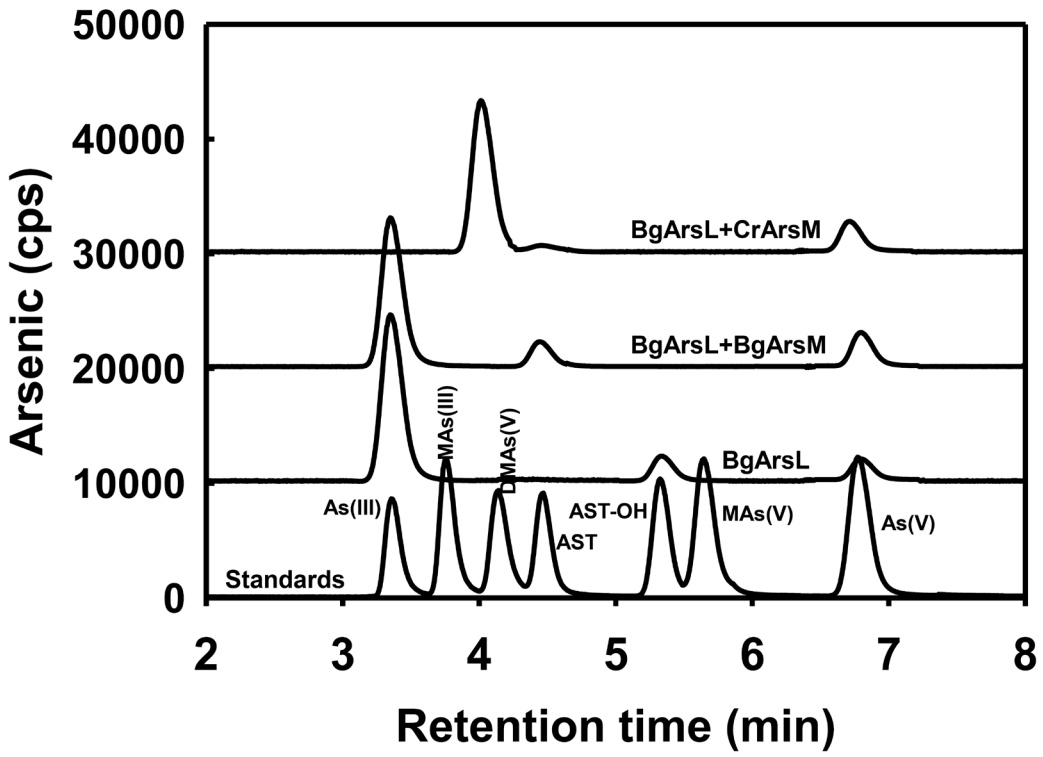


Figure 5. A BgArsM structural homology model A. Crystal structure of CmArsM (PDB: ID 4FR0) and **B. BgArsM homology model**. Both are shown as surface representations. The N-terminal domain that contains the SAM binding site is shown in green. The central and C-terminal domains are shown in cyan. The As(III) binding site is shown in yellow. The two halves

of the protein are connected by a short sequence in CmArsM, which is much larger in BgArsM because of the additional 37 residues (wheat). At the interface of the two halves of the protein is a cleft to which methylation inhibitors are proposed to bind (black rectangle).

BgArsM provides an evolutionary advantage for biosynthesis of the arsenic-containing antibiotic arsinothricin

What is the advantage of having an enzyme like BgArsM that methylates organoarsenicals but not As(III)? Together the *BgarsM* and *BgarsL* genes are part of the biosynthetic gene cluster for synthesis of the arsenic-containing antibiotic arsinothricin. The substrate of the radical SAM enzyme BgArsL are As(III) and SAM, which form trivalent R-AST-OH, the substrate of BgArsM. If As(III) were also a substrate of BgArsM, AST biosynthesis would be reduced because 1) the pool of available As(III) for BgArsL would be siphoned into MAs(III) synthesis, and 2) As(III) would competitively inhibit binding of R-AST-OH to BgArsM. In the microaerobic environment of the rice rhizosphere, MAs(III) would be oxidized and lose its antibiotic properties, while pentavalent AST would be a stable antibiotic. Thus, there is a clear evolutionary advantage for the antibiotic-synthesizing microbe to prevent As(III) methylation by BgArsM. To experimentally examine this possibility, we co-expressed BgArsL with either BgArsM or CrArsM in the pETDuet vector and assayed AST production (Fig 6). The results demonstrate that individually, BgArsL converted As(III) to AST-OH, while co-expression of BgArsL and BgArsM produced AST. In contrast, when BgArsL and CrArsM were co-expressed, the primary product was DMAs(V), and only a very small amount of AST was detected. These results demonstrate that BgArsM provides an evolutionary advantage for AST biosynthesis.



400

401 **Figure 6.** Comparison of the AST biosynthesis of cells co-expressing *BgarsL* with either *BgarsM*
402 or *CrarsM*. Cell of BL21(DE3) with the appropriate plasmids were grown in LB medium
403 overnight, transferred to R2A medium at a cell density of A_{600nm} = 3.0. Arsenical was then added
404 at 5 μM, final concentration, to the cell suspensions, which were incubated at 30 °C with
405 shaking for 6 h. Arsenic species in the medium were determined by HPLC-ICP-MS.

406

407 **ENVIRONMENTAL IMPLICATIONS**

408 Misuse and overuse of antibiotics worldwide accelerate antibiotic resistance and decrease their
409 efficacy, leading to one of the most critical public health threats.[36] Discovery and development of
410 new classes of antibiotics to prevent and reduce the emergence of antibiotic resistance are
411 urgently needed. Arsenic is a double-edged sword: at high concentrations it is a toxin and
412 poison, while at lower concentrations it has medicinal value.[37] For example, inorganic arsenic
413 trioxide (Trisenox), is a widely used chemotherapeutic drug used for treatment of acute

promyelocytic leukemia.[38] Aromatic arsenicals such as melarsoprol and roxarsone are antiprotozonan agents used for the treatment of trypanosomal diseases or prevention of coccidiosis in animal husbandry.[39] AST has the potential to be the first identified member of new classes of organoarsenical antibiotics. Elucidating how enzymes such as BgArsM and BgArsL catalyze AST biosynthesis provides insights into the evolution of antibiotic biosynthetic pathways.

Supporting Information. Plasmids, oligonucleotides and protein sequence alignments are supplied as Supporting Information

ACKNOWLEDGEMENTS

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Selective methylation by an ArsM S-adenosylmethionine methyltransferase from *Burkholderia gladioli* GSRB05 enhances antibiotic production

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Contents:

Table S1. Plasmids used in this study.

Plasmids	**Characteristics**	**Source**
pUC57-Kan-*BgarsM*	Km^r, clone vector containing synthesized *BgarsM* with 5' *Nco*I and 3' *Xho*I sites	GenScript, NJ, USA
pET28a(+)	Km^r, expression vector	Novagen
pETDuet-1	Amp^r, clone and expression vector, for the coexpression of two target genes	Millipore Sigma
pET28a-*BgarsM*	Km^r; *Nco*I-*Xho*I fragment containing *BgarsM* inserted into pET28a(+)	This study
pET28a-*CrarsM*	Km^r; *Nco*I-*Xho*I fragment containing *CrarsM* inserted into pET28a(+)	1
pET28a-*BgarsM*-C30S	Km^r; *Nco*I-*Xho*I fragment containing *BgarsM*-C30S inserted into pET28a(+)	This study
pET28a-*BgarsM*-C54S	Km^r; *Nco*I-*Xho*I fragment containing *BgarsM*-C54S inserted into pET28a(+)	This study
pET28a-*BgarsM*-C181S	Km^r; *Nco*I-*Xho*I fragment containing *BgarsM*-C181S inserted into pET28a(+)	This study
pET28a-*BgarsM*-C233S	Km^r; *Nco*I-*Xho*I fragment containing *BgarsM*-C233S inserted into pET28a(+)	This study
pET28a-$BgarsM_{Del}$	Km^r; *Nco*I-*Xho*I fragment containing $BgarsM_{Del}$ inserted into pET28a(+)	This study
pETDuet-*BgarsL*	Amp^r; *Sac*I - *Pst*I fragment containing *BgarsL* inserted into pETDuet-1	2
pETDuet-*BgarsL-BgarsM*	Amp^r; *Sac*I - *Pst*I fragment containing *BgarsL and NdeI-XhoI* fragment containing *BgarsM* inserted into pETDuet-1	2
pETDuet-*BgarsL-CrarsM*	Amp^r; *Sac*I - *Pst*I fragment containing *BgarsL and NdeI-XhoI* fragment containing *CrarsM* inserted into pETDuet-1	This study

Table S2. Primers used in the study

Primers	**Oligonucleotide sequence (5'-3'), restriction site**
Primers for vector construction	
pETDuet-*BgarsL-CrarsM*-F	CGCCATATGGTGGAGCCGGCTTCCATCGCG, *Nde*I
pETDuet-*BgarsL-CrarsM*-R	CCGCTCGAGTCAGCAGCAGGCGCCGCCGGG, *Xho*I
Primers for site-directed mutagenesis	
pET28a-*BgarsM*-C30S-F	GTACCAAGTTCTGAGCACCGCGGAGGC
pET28a-*BgarsM*-C30S-R	GCCTCCGCGGTGCTCAGAACTTGGTAC
pET28a-*BgarsM*-C54S-F	TGGACGCGGATTTCGGTAGCGGCAACC
pET28a-*BgarsM*-C54S-R	GGTTGCCGCTACCGAAATCCGCGTCCA
pET28a-*BgarsM*-C181S-F	GATGTGATTGTTAGCAACAGCGTGATCAACCTGGTTA
pET28a-*BgarsM*-C181S-R	TAACCAGGTTGATCACGCTGTTGCTAACAATCACATC
pET28a-*BgarsM*-C233S-F	TGTGGGCGGCGAGCTATGCGGGC
pET28a-*BgarsM*-C233S-R	GCCCGCATAGCTCGCCGCCCACA
pET28a-*BgarsM*-*Bgl*II-1-F	CATCACCGACCTGCGTACAGATCTGGAGAAG
pET28a-*BgarsM*-*Bgl*II-1-R	GTCCACCTTCTCCAGATCTGTACGCAGGTCGG
pET28a-*BgarsM*-*Bgl*II-2-F	TTAACGCGAAAGATCTGATTCCGGACAAC
pET28a-*BgarsM*-*Bgl*II-2-R	GATGCTGTTGTCCGGAATCAGATCTTTCGCGT

Figure S1. **Multiple alignment of BgArsM homologs.** The sequence of BgArsM from *B. gladioli* GSRB05 (WP_219608244) is compared with homologs. The protein source and GenBank accession numbers (in parentheses) of the aligned sequences are *Tepidicaulis marinus* (WP_052379435), *Homo sapiens* (NP_065733.2), *Chlamydomonas reinhardtii* (AFS88933.2), *Cyanidioschyzon* sp. 5508 (ACN39190.1), *Rhodopseudomonas palustris* (WP_011159102.1). Identities are highlighted in black, and conservative replacements in grey. The conserved cysteine residues and 37 amino acid residues insert are highlighted in yellow.

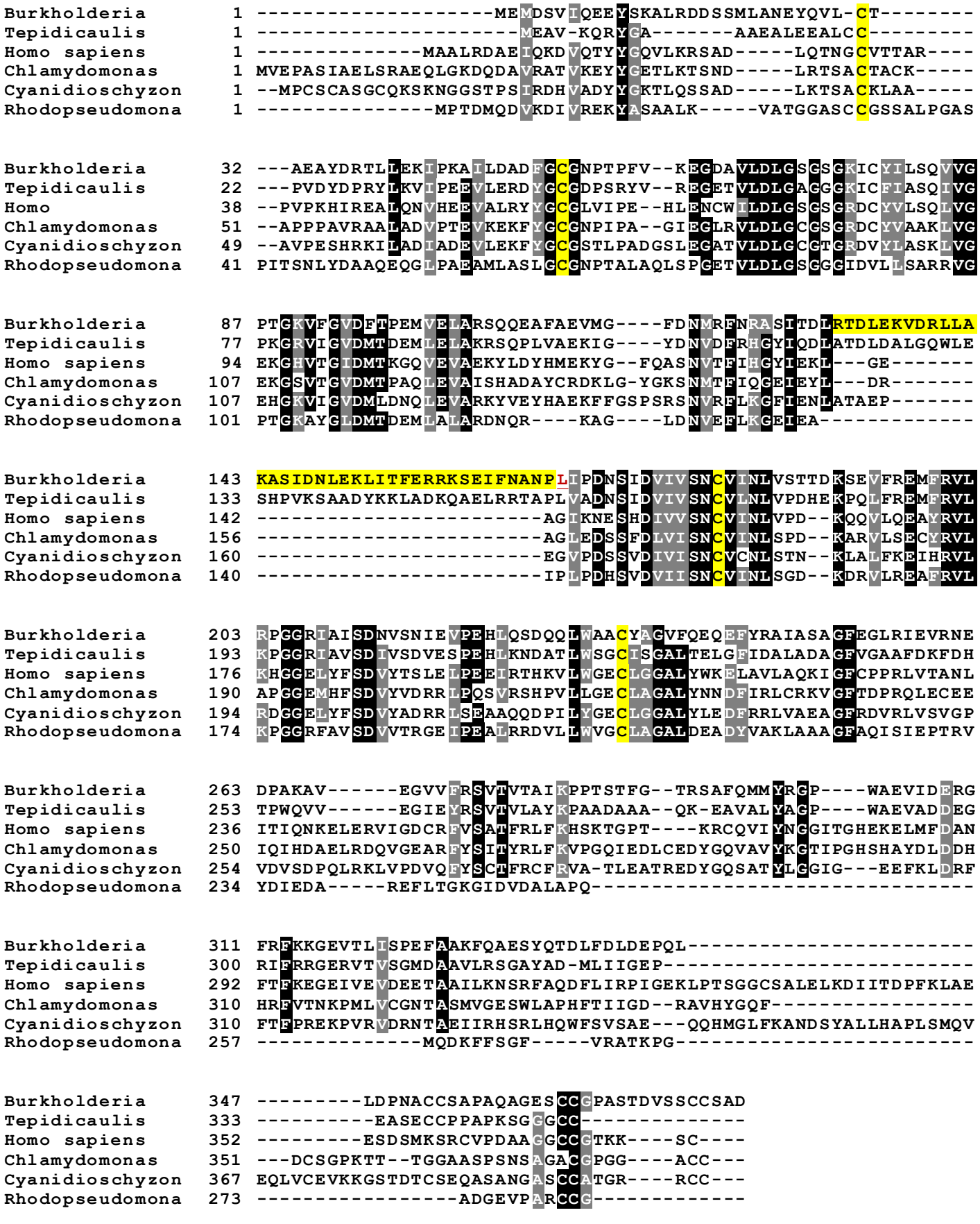
Burkholderia 1 ----------MEMDSVIQEEYSKALRDDSSMLANEYQVL-CT--------
Tepidicaulis 1 ----------MEAV-KQRYGA-------AAEALEEALCC---------
Homo sapiens 1 ----------MAALRDAEIQKDVQTYYGQVLKRSAD-----LQTNGCVTTAR----
Chlamydomonas 1 MVEPASIAELSRAEQLGKDQDAVRATVKEYYGETLKTSND-----LRTSACTACK-----
Cyanidioschyzon 1 --MPCSCASGCQKSKNGGSTPSIRDHVADYYGKTLQSSAD-----LKTSACKLAA-----
Rhodopseudomona 1 ----------MPTDMQDVKDIVREKYASAALK-----VATGGASCCGSSALPGAS
Burkholderia 32 ---AEAYDRTLLEKIPKAILDADFGCCGNPTPFV--KEGDAVLDLGSGSGKICYILSQVVG
Tepidicaulis 22 ---PVDYDPRYLKVIPEEVLERDYGCCGDPSRYV--REGETVLDLGAGGGKICFIASQIVG
Homo 38 --PVPKHIREALQNVHEEVALRYYGCCGLVIPE--HLENCWILDLGSGSGRDCYVLSQLVG
Chlamydomonas 51 --APPPAVRAALADVPTEVKEKFYGCCGNPIPA--GIEGLRVLDLGCGSGRDCYVAAKLVG
Cyanidioschyzon 49 --AVPESHRKILADIADEVLEKFYGCCGSTLPADGSLEGATVLDLGCGTGRDVYLASKLVG
Rhodopseudomona 41 PITSNLYDAAQEQGLPAEAMLASLGCCGNPTALAQLSPGETVLDLGSGGGIDVLLSARRVG
Burkholderia 87 PTGKVFGVDFTPEMVELARSQQEAFAEVMG----FDNMRFNRASITDLRTDLEKVDRLLA
Tepidicaulis 77 PKGRVIGVDMTDEMLELAKRSQPLVAEKIG----YDNVDFRHGYIQDLATDLDALGQWLE
Homo sapiens 94 EKGHVTGIDMTKGQVEVAEKYLDYHMEKYG--FQASNVTFIHGYIEKL---GE-------
Chlamydomonas 107 EKGSVTGVDMTPAQLEVAISHADAYCRDKLG-YGKSNMTFIQGEIEYL---DR-------
Cyanidioschyzon 107 EHGKVIGVDMLDNQLEVARKYVEYHAEKFFGSPSRSNVRFLKGFIENLATAEP-------
Rhodopseudomona 101 PTGKAYGLDMTDEMLALARDNQR----KAG----LDNVEFLKGEIEA-------------
Burkholderia 143 KASIDNLEKLITFERRKSEIFNANPLIPDNSIDVIVSNCVINLVSTTDKSEVFREMFRVL
Tepidicaulis 133 SHPVKSAADYKKLADKQAELRRTAPLVADNSIDVIVSNCVLNLVPDHEKPQLFREMFRVL
Homo sapiens 142 ------------------------AGIKNESHDIVVSNCVINLVPD--KQQVLQEAYRVL
Chlamydomonas 156 ------------------------AGLEDSSFDLVISNCVINLSPD--KARVLSECYRVL
Cyanidioschyzon 160 ------------------------EGVPDSSVDIVISNCVCNLSTN--KLALFKEIHRVL
Rhodopseudomona 140 ------------------------IPLPDHSVDVIISNCVINLSGD--KDRVLREAFRVL
Burkholderia 203 RPGGRIAISDNVSNIEVPEHLQSDQQLWAACYAGVFQEQEFYRAIASAGFEGLRIEVRNE
Tepidicaulis 193 KPGGRIAVSDIVSDVESPEHLKNDATLWSGCISGALTELGFIDALADAGFVGAAFDKFDH
Homo sapiens 176 KHGGELYFSDVYTSLELPEEIRTHKVLWGECLGGALYWKELAVLAQKIGFCPPRLVTANL
Chlamydomonas 190 APGGEMHFSDVYVDRRLPQSVRSHPVLLGECLAGALYNNDFIRLCRKVGFTDPRQLECEE
Cyanidioschyzon 194 RDGGELYFSDVYADRRLSEAAQQDPILYGECLGGALYLEDFRRLVAEAGFRDVRLVSVGP
Rhodopseudomona 174 KPGGRFAVSDVVTRGEIPEALRRDVLLWVGCLAGALDEADYVAKLAAAGFAQISIEPTRV
Burkholderia 263 DPAKAV------EGVVFRSVTVTAIKPPTSTFG--TRSAFQMMYRGP----WAEVIDERG
Tepidicaulis 253 TPWQVV------EGIEYRSVTVLAYKPAADAAA--QK-EAVALYAGP----WAEVADDEG
Homo sapiens 236 ITIQNKELERVIGDCRFVSATFRLFKHSKTGPT----KRCQVIYNGGITGHEKELMFDAN
Chlamydomonas 250 IQIHDAELRDQVGEARFYSITYRLFKVPGQIEDLCEDYGQVAVYKGTIPGHSHAYDLDDH
Cyanidioschyzon 254 VDVSDPQLRKLVPDVQEYSCTFRCFRVA-TLEATREDYGQSATYLGGIG---EEFKLDRF
Rhodopseudomona 234 YDIEDA-----REFLTGKGIDVDALAPQ--------------------------------
Burkholderia 311 FRFKKGEVTLISPEFAAKFQAESYQTDLFDLDEPQL------------------------
Tepidicaulis 300 RIFRRGERVTVSGMDAAVLRSGAYAD-MLIIGEP-------------------------
Homo sapiens 292 FTFKEGEIVEVDEETAAILKNSRFAQDFLIRPIGEKLPTSGGCSALELKDIITDPFKLAE
Chlamydomonas 310 HRFVTNKPMLVCGNTASMVGESWLAPHFTIIGD--RAVHYGQF-----------------
Cyanidioschyzon 310 FTFPREKPVRVDRNTAEIIRHSRLHQWFSVSAE---QQHMGLFKANDSYALLHAPLSMQV
Rhodopseudomona 257 --------------MQDKFFSGF-----VRATKPG------------------------
Burkholderia 347 ---------LDPNACCSAPAQAGESCCGPASTDVSSCCSAD
Tepidicaulis 333 ----------EASECCPPAPKSGGGCC--------------
Homo sapiens 352 ---------ESDSMKSRCVPDAAGGCCGTKK----SC----
Chlamydomonas 351 ---DCSGPKTT--TGGAASPSNSAGACGPGG----ACC---
Cyanidioschyzon 367 EQLVCEVKKGSTDTCSEQASANGASCCATGR----RCC---
Rhodopseudomona 273 -----------------ADGEVPARCCG-------------

Figure S2. Multiple alignment the sequence of the insert in BgArsM with the 100 closest orthologs. The protein sequence of BgArsM from *B. gladioli* GSRB05 (WP_219608244) was used for blastp (protein-protein BLAST) in the NCBI blastp suite (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Shown is the alignment of BgArsM residues 154 to 203 with the 100 closest orthologs.

References:

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