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Reduction of organoarsenical herbicides and antimicrobial growth promoters by the legume symbiont *Sinorhizobium meliloti*

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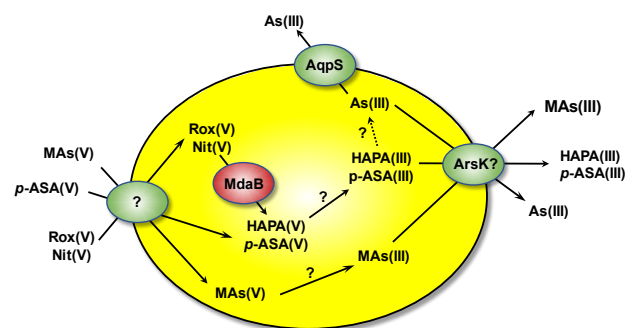
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21 **Running title:** Bacterial reduction of environmental organoarsenicals



Sinorhizobium meliloti Rm1021

Abstract: Massive amounts of methyl (e.g. methylarsenate, MAs(V)) and aromatic arsenicals (e.g. roxarsone (4-hydroxy-3-nitrophenylarsonate, Rox(V)) have been utilized as herbicides for weed control and growth promoters for poultry and swine, respectively. The majority of these organoarsenicals degrade into more toxic inorganic species. Here we demonstrate that the legume symbiont *Sinorhizobium meliloti* both reduces MAs(V) to MAs(III) and catalyzes sequential two-step reduction of nitro and arsenate groups in Rox(V), producing the highly toxic trivalent amino aromatic derivative 4-hydroxy-3-aminophenylarsenite (HAPA(III)). The existence of this process suggests that *S. meliloti* evolved the ability to transform pentavalent methyl and aromatic arsenicals into antibiotics to provide a competitive advantage over other microbes, which would be a critical process for the synthetic aromatic arsenicals to function as antimicrobial growth promoters. The activated trivalent aromatic arsenicals are degraded into less toxic inorganic species by an MAs(III)-demethylating aerobe, suggesting that environmental aromatic arsenicals also undergo a multiple-step degradation pathway, in analogy with the previously-reported demethylation pathway of methylarsenate herbicide. We further show that an FAD-NADPH-dependent nitroreductase encoded by *mdaB* gene catalyzes nitroreduction of roxarsone both *in vivo* and *in vitro*. Our results demonstrate that environmental organoarsenicals trigger competition between members of microbial communities, resulting in gradual degradation of organoarsenicals and contamination by inorganic arsenic.

INTRODUCTION

The metalloid arsenic, the most pervasive environmental toxin, has exerted selective pressure on organisms since life emerged on Earth, providing a selective pressure for the evolution and acquisition of arsenic resistance mechanisms in nearly every extant organism. Bacteria especially have developed a variety of unique arsenic resistance (*ars*) genes^{1,2}. One such gene is *arsM*, which encodes the ArsM As(III) S-adenosylmethionine methyltransferase³. ArsM evolved before the Great Oxidation Event (GOE) and generates reduced methylarsenicals such as methylarsenite (MAs(III)) and dimethylarsenite (DMAs(III))^{4,5}. These trivalent methylated arsenic species are much more toxic than the inorganic forms and stable in anoxic environments, and we have proposed that these ancient microbes activated available arsenic into more toxic forms for use as antibiotics to kill competitors^{4,6}. In the present oxic biosphere, arsenic methylation primarily functions as a detoxification mechanism because trivalent methylarsenicals are rapidly oxidized to relatively nontoxic methylarsenate (MAs(V)) and dimethylarsenate (DMAs(V))³. In response, other bacteria revived the antibiotic strategy by re-reducing MAs(V) to the trivalent form. Other bacteria answered back by evolving multiple mechanisms to reverse the antibiotic action of MAs(III): by oxidation, demethylation or extrusion^{4,6}.

Methylated arsenic species are introduced into the environment not only through biogenic sources but also as a result of anthropogenic activities. In the United States, massive amount of

63 methylarsenicals, both MAs(V) and DMAs(V), have been utilized as herbicides since the early
64 1970s. Although the use of DMAs(V) has been prohibited since the beginning of 2014 under the
65 cancellation order issued by the U.S. Environmental Protection Agency (EPA), the monosodium
66 salt of MAs(V), or MSMA, is still allowed for use on cotton fields, golf courses, sod farms and
67 highway rights of way nationwide except in Florida, where the use is now banned in golf
68 courses⁷.

69
70 In addition to MSMA, synthetic pentavalent aromatic arsenicals, including Rox(V), nitarsons
71 (4-nitrophenylarsenate; Nit(V)) and *p*-arsanilic acid (4-aminophenylarsenate; *p*-ASA(V)) have
72 been heavily used since the 1940s, especially in the poultry industry, as a feed additive to control
73 protozoan parasitic diseases and promote growth. Approximately 2,000,000 pounds of Rox(V)
74 was annually released into the environment as manure from Rox(V)-fed animals and the manure
75 was applied as fertilizer to farm crops⁸. Although use of these aromatic arsenical growth
76 promoters is no longer allowed in the United States
77 ([https://www.fda.gov/animal-veterinary/product-safety-information/arsenic-based-animal-drugs-a](https://www.fda.gov/animal-veterinary/product-safety-information/arsenic-based-animal-drugs-and-poultry)
78 [nd-poultry](https://www.fda.gov/animal-veterinary/product-safety-information/arsenic-based-animal-drugs-and-poultry)), these compounds are still in use in other countries⁹. Large portion of these
79 anthropogenic organoarsenic compounds gradually degrade into more toxic inorganic forms
80 through microbial activities that impact the environment and contaminate our water and food
81 supplies^{10–12}. A number of bacterial species, both aerobes^{9,13–15} and anaerobes^{16–18}, have been

documented to degrade methyl and aromatic arsenicals, however, the pathways and molecular mechanisms for degradation of organoarsenicals remain poorly understood.

We previously identified a microbial community from Florida golf course soil that degrades MAs(V) aerobically¹⁹, where one microbe activates MAs(V) into highly toxic MAs(III) that other member subsequently detoxifies into less toxic inorganic As(III). The MAs(III) demethylation is catalyzed by the Arsl C-As lyase, an Fe(II)-dependent dioxygenase²⁰. Here we demonstrate that pentavalent aromatic arsenicals are also biotransformed into inorganic species via sequential reduction and Arsl-catalyzed C-As bond cleavage by aerobic microbial communities, similar to the pathway of demethylation of the MSMA herbicide. Legume symbiont *Sinorhizobium meliloti* Rm1021 is a typical soil bacterium and forming nitrogen-fixing symbiosis with alfalfa and other legumes. *S. meliloti* Rm1021 has been extensively studied as a model organism for the genetic analysis of symbiotic nitrogen fixation and legume-microbe interaction. The sequencing of the entire genome of *S. meliloti* Rm1021 has been completed in 2001²¹. For As metabolism, *S. meliloti* Rm1021 can utilize a unique detoxification pathway wherein reduction of As(V) by arsenate reductase (ArsC) is coupled to downhill transport of As(III) through the aquaglyceroporin (AqpS) channel²². In this study, we demonstrate that the *S. meliloti* Rm1021 reduces both MAs(V) and pentavalent aromatic arsenicals, producing trivalent methyl and aromatic arsenicals. Other Rox(V)-degrading aerobes⁹ and anaerobes^{16–18} have been reported

to reduce the nitro group of nitroaromatic arsenicals such as Rox(V) and Nit(V). We found that *S. meliloti* Rm1021 also reduces the nitro groups of Rox(V) and Nit(V) to form the corresponding aromatic amines 4-hydroxy-3-aminophenylarseate (HAPA(V)) and *p*-ASA(V), respectively. The arsenic in these species is subsequently reduced, producing trivalent HAPA(III) and *p*-ASA(III), which are much more toxic compared to the corresponding pentavalent species²³. When cells of *S. meliloti* Rm1021 were co-cultured with *Streptomyces* sp. MD1, an MAs(III)-demethylating species isolated from Florida golf course soil¹⁹, degraded both methyl and aromatic pentavalent arsenicals into inorganic As(III). Further, we identified *mdaB*, the gene for the first reduction step, nitroreduction of nitroaromatic arsenicals, and characterized the gene product MdaB both *in vivo* and *in vitro*.

METHODS AND MATERIALS

Strains and chemicals

S. meliloti Rm1021²⁴ and its derivative strain RmP310 that lacks the chromosomal *ars* operon (Δ *ars*)^{23,25}, *Pseudomonas putida* KT2440²⁶, *Burkholderia* sp. MR1 and *Streptomyces* sp. MD1¹⁹ were used to analyze biotransformation and uptake of organoarsenicals. *Escherichia coli* strain TOP10 (Invitrogen, Waltham, MA) was used for plasmid construction and replication. *E. coli* strains AW3110(DE3) (Δ *ars*)²⁷ and BL21(DE3) (Novagen, Madison, WI) were used for analysis of activity of nitroreductases *in vivo* and protein expression, respectively. Except where specified,

bacterial strains were cultured aerobically with shaking at 37 °C (*E. coli* strains) or 30 °C (others). Unless otherwise indicated, all chemicals were of analytical or better grade from MilliporeSigma (Burlington, MA). Rox(V) and HAPA(V) were purchased from Thermo Fisher Scientific (Waltham, MA) and Pfaltz & Bauer (Waterbury, CT), respectively. The trivalent organoarsenicals (Rox(III), Nit(III), *p*-ASA(III) and HAPA(III)) were prepared by chemical reduction of their corresponding pentavalent forms as previously described²⁰. In brief, 0.2 mM pentavalent arsenical was mixed with 27 mM Na₂S₂O₃, 66 mM Na₂S₂O₅, and 82 mM H₂SO₄, following which the pH was adjusted to 6 with NaOH. We confirmed by absorption spectroscopy that the chemical reduction method reduces As(V) to As(III) but does not reduce the nitro group of roxarsone and nitarsonic acid. The methylarsonous acid iodide derivative (MAs(III)I₂) synthesized as described²⁸ was used as MAs(III). For confirmation purpose, prepared MAs(III)I₂, *p*-ASA(III) and HAPA(III) were treated with 4.5 % (v/v) H₂O₂ and incubated first for 10 min at room temperature and further for 5 min at 80 °C. Oxidized organoarsenicals were analyzed by HPLC-ICP-MS as described below to confirm they match the corresponding pentavalent species (Fig. S1A, C and F). The chemical structures of arsenicals are shown in Fig. S2.

Biotransformation of orerganoarsenicals

S. meliloti Rm1021, *S. meliloti* RmP310 (Δ ars), *Burkholderia* sp. MR1 and *P. putida* KT2440 were solely cultured with 1 μ M MAs(V), *p*-ASA(V), Nit(V) or Rox(V) in ST 10⁻¹ medium²⁹ supplemented

139 with 0.2% D-glucose, 2.5 mM CaCl₂ and 2.5 mM MgSO₄ for 3 days. As shown by our present
140 results, *S. meliloti* Rm1021 displayed an activity to reduce MAs(V), *p*-ASA(V), Nit(V) and Rox(V)
141 while it had a poor ability to degrade them into As(III). Our previous study has shown that a C-As
142 lyase (ArsI) is responsible for degradation of trivalent organoarsenicals into As(III)²⁰. Therefore,
143 we co-cultured *S. meliloti* Rm1021 with *Streptomyces* sp. MD1, a soil bacterium isolated from
144 golf course soils that contains *arsI* gene in its genome¹⁹⁻²⁰, to investigate the complete
145 degradation pathway of pentavalent organoarsenicals into inorganic arsenic. For co-culture
146 experiments, *S. meliloti* Rm1021 and *Streptomyces* sp. MD1 were first cultured separately for 24
147 hours and then co-cultured in the presence of pentavalent organoarsenicals for an additional 3
148 days. We independently repeated the bacterial culture/co-culture experiment three times. Each
149 culture was centrifuged at 13,400 g for 1 min, and the supernatant solution was immediately
150 filtered by Amicon Ultra Centrifugal Filters with a 3,000 Da cut-off membrane (MilliporeSigma).
151 The arsenic species in the filtrates were determined by high-performance liquid chromatography
152 (HPLC) (Series 2000; PerkinElmer, Waltham, MA) coupled to Inductively coupled plasma mass
153 spectrometry (ICP-MS) (ELAN DRC-e; PerkinElmer) using the previous instrument parameters²⁰.
154 Briefly, MAs(V), *p*-ASA(V), HAPA(V), and their trivalent forms were analyzed with Thermo Fisher
155 Scientific BioBasic™ 18 LC column (250 mm × 4.6 mm, 5 μm, 300 Å) isocratically eluted with a
156 mobile phase consisting of 3 mM malonic acid and 5% methanol (v/v) (pH 5.95 for MAs and 5.2
157 for *p*-ASA and HAPA, adjusted by tetrabutylammonium hydroxide), with a flow rate of 1 mL min⁻¹
158 at 25 °C. Nit(V), Rox(V) and their trivalent forms were analyzed with Inertsil C4 column (150 mm

× 2.1 mm; 5 µm; GL Sciences, Japan) isocratically eluted with a mobile phase consisted with 15% acetonitrile (v/v), 5% ethanol (v/v) and 80% water (pH 1.5 adjusted by HCl) (v/v), at a flow rate of 0.3 or 0.6 mL min⁻¹ at 60 °C. For confirmation purpose, samples containing MAs(III), *p*-ASA(III) and HAPA(III) were treated with 4.5 % (v/v) H₂O₂ and incubated first for 10 min at room temperature and further for 5 min at 80 °C. Oxidized samples were analyzed by HPLC-ICP-MS to confirm their species (Fig. S1B, D, E and G). Each amount of the indicated arsenic species in bacterial culture/co-culture samples was quantified from the corresponding peak area using Chromera Chromatography Data System version 2.1 (Perkin Elmer) according to standard curves prepared with standard solutions in the range of 0.5-2 µM in water. The results from the triplicated independent experiments are summarized in Table S1.

Plasmid construction

By conducting a BLAST (Basic Local Alignment Search Tool) search of the genome of *S. meliloti* 1021, the genes encoding SmAzoR (NCBI accession No.: NP_385442), SmMdaB (NP_387022), SmMsuE (NP_438025) and SmNemA (NP_385670), SmAZR (NP_386600) and SmNitB (NP_384119) were chosen as candidates responsible for the nitroreduction of roxarsone and nitarosone (See *Results and discussion*). For construction of plasmids for expression of *SmazoR*, *SmnemA* and *SmnitB*, each gene was PCR-amplified from total genomic DNA of *S. meliloti* Rm1021 with *PfuTurbo* DNA polymerase (Agilent Technologies Inc., Santa Clara, CA) using the

forward and reverse primers listed in Table S2. Amplicons were digested by *NheI* and *HindIII* (*SmazoR*) or *NcoI* and *XhoI* (*SmnemA* and *SmnitB*) and ligated into pET28a, generating the plasmids pET28a-*SmazoR*, pET28a-*SmnemA* and pET28a-*SmnitB*. The genes of *SmmdaB*, *SmmsuE* and *Smazr* were chemically synthesized with 5' *NcoI* and 3' *XhoI* sites and cloned into pET28a by GenScript Biotech Corp. (Piscataway, NJ), generating the plasmids pET28a-*SmmdaB*, pET28a-*SmmsuE* and pET28a-*Smazr*.

***In vivo* nitroreduction of trivalent nitroaromatic arsenicals**

In vivo nitroreduction of Rox(III) by *E. coli* AW3110(DE3) cells carrying the constructed plasmids was analyzed spectrophotometrically, as described previously³⁰. Briefly, After overnight growth in LB medium supplemented with 25 $\mu\text{g mL}^{-1}$ kanamycin and 0.3 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) at 30 °C, the cells were washed once with low phosphate medium³¹ supplemented with 0.2% D-glucose and suspended in glucose-free M9 medium³² at a cell density of $A_{600\text{ nm}} = 10.0$. The cell suspensions were then incubated with 60 μM Rox(III) at 30 °C with shaking (200 rpm) for 6 h. The nitroreductase activity of cells was estimated by loss of absorbance at 410nm determined with a Synergy H4 Hybrid Multi-Mode microplate reader (BioTek Instruments, Inc., Winooski, VT). *In vivo* nitroreductase activity of SmMdaB was also analyzed by arsenic speciation. Cell suspensions of *E. coli* AW3110(DE3) cells harboring pET28a-*SmmdaB* prepared at a cell density of $A_{600\text{ nm}} = 2.0$ in the same way were cultured with 4

197 μ M Rox(III) or Nit(III) at 30 °C with shaking for indicated hours and arsenic species in the culture
198 medium were determined by HPLC-ICP-MS as described above.

200 **Protein purification**

201 *E. coli* BL21(DE3) cells harboring pET28a-*SmmdaB* were grown in LB medium containing 50 mg
202 L⁻¹ kanamycin. The cells at an A_{600 nm} of 0.6 were induced by 0.3 mM IPTG and further cultured
203 for 4 h. The cells were then harvested, suspended in 5 mL per gram of wet cells in Buffer A (50
204 mM 4-morpholinepropanesulfonic acid, 20 mM imidazole, 0.5 M NaCl, 10 mM
205 2-mercaptoethanol and 20% glycerol (vol/vol), pH 7.5), lysed by a single passage through a
206 French pressure cell at 20,000 psi and treated with 2.5 μ L of diisopropyl fluorophosphate per
207 gram of wet cells. After centrifugation at 150,000 x g for 1 h, the resultant supernatant solution
208 was loaded onto a Ni²⁺-nitrilotriacetic acid column (Qiagen, Hilden, Germany) at a flow rate of 0.5
209 mL min⁻¹. The column was washed with more than 25 column volumes of Buffer A. His-tagged
210 SmMdaB was eluted with Buffer A with increased concentration of imidazole (0.2 M) and the
211 purity was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Protein
212 concentrations were estimated from A_{280 nm} (ϵ = 53,910 M⁻¹·cm⁻¹). SmMdaB-containing fractions
213 were divided into small portions, rapidly frozen, and stored at -80 °C until use.

215 **FAD reductase activity**

The amount of flavin adenine dinucleotide (FAD) in purified SmMdaB was quantified by absorption at 450 nm using a molar extinction coefficient of $11,300 \text{ M}^{-1}\cdot\text{cm}^{-1}$. Reduction of FAD by SmMdaB was assayed in 25 mM Bis-Tris propane buffer (pH 7.0) containing 1 mM EDTA and 0.1 mg mL^{-1} bovine serum albumin (BSA) at 37 °C. 0.2 mM NADPH was incubated with 1 μM SmMdaB and/or 25 μM FAD and the oxidation of NADPH was monitored by the decrease in absorbance at 340 nm ($\epsilon = 6,220 \text{ M}^{-1}\cdot\text{cm}^{-1}$).

***In vitro* nitroreduction of pentavalent nitroaromatic arsenicals**

Nitroreductase activity of SmMdaB was examined *in vitro* using purified protein. 4 μM of Rox(V) or Nit(V) was incubated at 37 °C in the presence or absence of 1 μM SmMdaB in a reaction solution (25 mM Tris, 25 mM Bis-Tris propane (pH 7.0), 1 mM EDTA, 0.1 mg mL^{-1} BSA, 0.2 mM NADPH and 25 μM FAD) with or without 5 mM glutathione (GSH) and/or 1 mM tris-(2-carboxyethyl)phosphine (TCEP). Reactions were collected at the indicated times and the arsenic species were analyzed by HPLC-ICP-MS, as described above.

Results and discussion

Reduction of MAs(V) by *S. meliloti*

The pure culture of *S. meliloti* Rm1021 is able to reduce MAs(V) to MAs(III) while the pure culture

of *Streptomyces* sp. MD1 cannot transform MAs(V) (Fig. 1A, Table S1). In contrast, co-cultures of *S. meliloti* Rm1021 and the MAs(III)-demethylating *Streptomyces* sp. MD1 degrade MAs(V) to inorganic As(III), which is consistent with our concept that microbial communities are capable of sequentially reducing MAs(V) to MAs(III), and demethylating the MAs(III) to As(III).

Reduction of aromatic arsenicals by *S. meliloti*

We previously identified the *arsI* gene for MAs(III) demethylation from *Bacillus* sp. MD1, an MAs(III)-demethylating bacterial isolate from Florida golf course soil²⁰. Cells of the arsenic-hypersensitive *Escherichia coli* strain AW3110²⁷ expressing *arsI* degrade trivalent aromatic arsenicals, including *p*-ASA(III), Nit(III) and Rox(III), into inorganic As(III), suggesting that Arsl also catalyzes aerobic breakdown of these antimicrobial animal growth promoters. It seemed reasonable to consider that pentavalent aromatic arsenicals also undergo sequential reduction and Arsl-catalyzed C-As bond cleavage by microbial communities. This is formally similar to demethylation of the MSMA herbicide by the MAs(V)-reducing *Burkholderia* sp. MR1 and the MAs(III)-demethylating *Streptomyces* sp. MD1 from Florida golf course soil. However, neither of *Burkholderia* sp. MR1 nor *Pseudomonas putida* KT2440, the other known MAs(V) reducer, are able to reduce pentavalent aromatic arsenicals (Fig. S3). In contrast, *S. meliloti* Rm1021 reduces them to the trivalent species. As with MAs(V), *S. meliloti* Rm1021 converts most of the *p*-ASA(V) to trivalent *p*-ASA(III) after 3 days (Fig. 1B, Table S1). On the other hand,

253 cells of *S. meliloti* Rm1021 transformed Nit(V) to primarily *p*-ASA(III) rather than Nit(III) (Fig. 1C
 254 and D, Table S1). A time-course analysis shows that *S. meliloti* Rm1021 first reduces the nitro
 255 group of Nit(V), forming *p*-ASA(V) (Fig. S4A and B, 36h), and subsequently reduces the arsenate
 256 group of *p*-ASA(V), forming *p*-ASA(III) (Fig. S4B, 48 and 60h). Similarly, *S. meliloti* Rm1021
 257 transformed Rox(V) to primarily HAPA(III) rather than Rox(III) (Fig. 1E and F, Table S1). The
 258 strain first reduces the nitroaromatic group of Rox(V), transforming it into the pentavalent amine
 259 derivative 4-hydroxy-3-aminophenylarsonate (HAPA(V)) (Fig. S4C and D, 36h), and
 260 subsequently reduces the As(V) moiety, forming HAPA(III) (Fig. S4D, 60h). Two unknown
 261 species are also produced from Rox(V) (Fig. S4C and D) but not Nit(V) (Fig. S4A and B). In
 262 addition, cells of *S. meliloti* Rm1021 produced small amounts of As(III) over time from both Nit(V)
 263 (Fig. S4B) and Rox(V) (Fig. S4D). Since *S. meliloti* Rm1021 does not have an *arsI* gene, As(III)
 264 might be a minor secondary product of reduction of aromatic arsenicals and/or adventitious C-As
 265 bond cleavage by other lyases such as C-P (carbon-phosphorus) lyases³³. In contrast, neither
 266 *Burkholderia* sp. MR1 nor *P. putida* KT2440 reduce either the nitro group or As(V) group (Fig. S3).
 267 The levels of the pentavalent aromatic arsenicals in the cultures of *Burkholderia* sp. MR1 and *P.*
 268 *putida* KT2440 are comparable to those in culture medium without cells, suggesting that these
 269 strains may not be able to take up pentavalent aromatic arsenicals. As is the case of MAs(V), a
 270 mixed culture of *S. meliloti* Rm1021 and *Streptomyces* sp. MD1 could degrade *p*-ASA(V) (Fig.
 271 1B), Nit(V) (Fig. 1D) and Rox(V) (Fig. 1F) into As(III). Given that *S. meliloti* is a ubiquitous
 272 rhizosphere soil bacterium³⁴ and that *arsI* genes are widely distributed from thermophiles³⁵ to

cyanobacteria³⁶, we propose that this multiple-step aromatic arsenical degradation pathway exists in the oxic environment and forms a part of the arsenic biogeochemical cycle.

Identification of the gene for nitroreduction of aromatic arsenicals

Since *S. meliloti* 1021 first reduces the nitro group and subsequently reduces the arsenate moiety (Fig. 1D and F), we consider the possibility that each reduction uses a different enzyme. Nitroreduction of aromatic arsenicals has been observed in anaerobic degradation^{17,37,38}, indicating that nitroreduction is a critical step in the anaerobic degradation of environmental aromatic arsenicals. The *undA* and *mtrC* genes, which are involved in iron reduction in *Shewanella putrefaciens* W3-18-1 strain³⁹, have been suggested to account in part for anaerobic reduction of Rox(V) to HAPA(V) by *S. putrefaciens* CN-32¹⁷. From *S. putrefaciens* 200, the highly versatile facultative anaerobe carrying a large arsenic island with a number of genes in several *ars* operons¹⁸, we recently found a novel bacterial resistance mechanism for trivalent nitroaromatic arsenicals that is also initiated with nitroreduction³⁰. Three linked genes widely distributed in *ars* operons from anaerobes, named *arseFG*, confers resistance to Nit(III) and Rox(III) by a combination of nitroreduction of Nit(III) or Rox(III) to *p*-ASA(III) or HAPA(III) by ArsEF and efflux of *p*-ASA(III) or HAPA(III) by ArsG.

In contrast, no molecular details are known for nitroreduction of aromatic arsenicals by aerobes.

Possibilities include known bacterial nitroreductases and azoreductases that have central roles in reduction of nitroaromatic compounds^{40,41}. Although their physiological roles remain unclear, these enzymes have gained considerable attention because of their potential applications in cancer treatment and bioremediation. A number of bacterial nitroreductases and azoreductases have been identified to activate nitroaromatic anticancer prodrugs such as CB1954 (5-(aziridine-1-yl)-2,4-dinitrobenzamide) and PR-104A (2-((2-bromoethyl)-2-[[2-(2-hydroxyethyl)amino]carbonyl]-4,6-dinitroanilino)ethyl methanesulfonate)⁴²⁻⁴⁴ and nitroaromatic antimicrobial prodrugs nitrofurans such as nitrofurazone and nitrofurantoin^{45,46} and to degrade nitroaromatic environmental contaminants such as 2,4,6-trinitrotoluene (TNT)⁴⁷⁻⁴⁹. Because these nitroaromatic compounds have similarities with Rox(V) and Nit(V), it was reasonable to consider that some are capable of reduction of Nit(V) and Rox(V). One candidate is ArsH, which physiologically oxidizes MAs(III) to MAs(V) for detoxification²¹. ArsH from *Pseudomonas aeruginosa* PAO1 has been demonstrated to reduce a nitroaromatic antibiotic (nitrofurazone) to the hydroxylamine derivative⁴⁶. However, a *S. meliloti* Rm1021 *ars* operon deletion strain lacking *arsH* (Δars)^{23,25} retains the ability to reduce nitroaromatic arsenicals (Fig. S5), indicating that little or no contribution of SmArsH. We conducted a BLAST search of the genome of *S. meliloti* 1021 to identify orthologs of AzoR, MdaB, MsuE and NemA enzymes that reduce CB1954 and/or PR-104A to hydroxylamine derivatives⁴²⁻⁴⁴. These include SmAzoR (NCBI accession No.: NP_385442), SmMdaB (NP_387022), SmMsuE (NP_438025) and SmNemA (NP_385670). There are also homologs of

AZR and NitB, enzymes that reduce TNT to hydroxylamino-dinitrotoluene^{48,49}, including SmAZR (NP_386600) and SmNitB (NP_384119).

To examine their potential role in reduction of nitroaromatic arsenicals, each of the above genes were cloned or synthesized and expressed in *E. coli* AW3110. Some bacteria, including *E. coli*, take up Rox(III) more effectively than Rox(V)^{18,30}, so reduction of Rox(III) was examined *in vivo*. Both pentavalent and trivalent roxarsone absorbs with a λ_{\max} = 410 nm at physiological pH, whereas the corresponding HAPA amine is colorless, which allows nitroreduction to be monitored by loss of absorption at 410 nm (Fig. S6). A significant decrease in $A_{410\text{nm}}$ was observed only in the cultures of cells expressing *SmmDaB*, so we focused on SmMdaB for further characterization. Rox(III) biotransformation by cells expressing *SmmDaB* was analyzed by HPLC-ICP-MS (Fig. 2). In the culture medium of these cells Rox(III) was mostly converted to HAPA(III), whereas no HAPA was produced in the medium of the control cells. The results are consistent with SmMdaB reduction of roxarsone to HAPA. In T24 human bladder carcinoma cells the IC₅₀ values for Rox(III) and HAPA(III) are 0.2 and 22 μM , respectively⁵⁰, suggesting that nitroreduction of Rox(III) to HAPA(III) could be a detoxification process. However, cells expressing *SmmDaB*, which transformed Rox(III) to HAPA(III), were not resistant to Rox(III) (data not shown), suggesting that HAPA(III) is also very toxic to *E. coli* and nitroreduction alone does not detoxify Rox(III) in the *E. coli* cells.

SmMdaB is nitroaromatic arsenical nitroreductase

MdaB (modulator of drug activity B) has been shown to be an FAD- and NADPH-dependent quinone reductase with nitroreductase activity with various nitroaromatic compounds^{43,46}. Nitroreduction by the *S. meliloti* ortholog SmMdaB was characterized *in vitro* using purified protein. Purified SmMdaB is yellow and exhibits an absorption spectrum with λ_{max} at 450 nm due to 61.2 % FAD occupancy. However, it still requires supplementation with FAD in addition to NADPH to exhibit FAD reductase activity (Fig. S7). SmMdaB requires an additional reducing potential such as GSH or TCEP for nitroreductase activity with Rox(V) in addition to FAD and NADPH (Fig. 3A). The added reductant may be required to reduce the nitro group all the way to the amine. In contrast, purified MdaB from *P. aeruginosa* PAO1 (PaMdaB) catalyzes nitroreduction of nitrofranzone with only NADPH as reductant *in vitro*, but the product is the hydroxylamine derivative, not the amine⁴⁶. No nitroreduction was observed without enzyme (Fig. 3B). SmMdaB also reduces nitarsonic acid to *p*-ASA both *in vivo* (Fig. S8) and *in vitro* (Fig. S9). Altogether, our results clearly demonstrate that SmMdaB is nitroaromatic arsenical nitroreductase.

Since *S. meliloti* Rm1021 first reduces the nitro group of Rox(V) and Nit(V) to form HAPA(V) and *p*-ASA(V) before reducing the arsenate moiety, neither of Rox(III) nor Nit(III) are produced

350 throughout the entire transformation process (Fig. S4A and C). We propose that nitroreduction by
351 SmMdaB may be required for subsequent reduction of the pentavalent arsenic. *Burkholderia* sp.
352 MR1 has no MdaB homolog. Although *P. putida* KT2440 has one MdaB homolog (PpMdaB) that
353 shares 41% identities and 59% positives with SmMdaB, the strain exhibits no nitroreductase
354 activity with nitroaromatic arsenicals, perhaps due to the poor uptake of those compounds (Fig.
355 S3B and C). BLAST analyses show that none of the reported Rox(V)-degrading anaerobes such
356 as *Alkaliphilus oremlandii*¹⁶ and *Shewanella putrefaciens*^{17,18} carry *mdaB* genes, whereas
357 *Enterobacter* sp. CZ-1, the only reported Rox(V)-transforming aerobe⁹, possesses multiple *mdaB*
358 genes and the gene products share ~40% identities and ~60% positives with SmMdaB,
359 suggesting that MdaB plays a role in nitroreduction of Rox(V) and Nit(V) in that organism.
360 However, MdaB homologs from *E. coli* (EcMdaB) and *P. syringae* (PsMdaB) share 60% identities
361 and 74% positives, nevertheless, only EcMdaB exhibits significant nitroreductase activity with
362 PR-104A⁴⁴, indicating that even minor differences in protein sequences could account for the
363 substrate selectivity of different MdaBs. The *mdaB* genes are widely distributed among both
364 Gram-positive and Gram-negative bacteria. Structure-function analyses are required to elucidate
365 the determinants of substrate selectivity. This information will shed light on the impact of MdaB
366 on the fate of environmental nitroaromatic arsenicals. In contrast to nitroreduction, the molecular
367 mechanisms of reduction of arsenate group in aromatic arsenicals are completely unknown.
368 Further studies are required to identify new gene(s)/protein(s) involved in the arsenate reduction
369 of the growth promoters.

370

371 **Environmental implication**

372 With the exception of only a few natural products as typified by chloramphenicol, most
373 nitroaromatic compounds, including roxarsone and nitarsones, are produced and released into
374 the environment via industrial processes or other human activities⁴⁰. Synthetic nitroaromatic
375 compounds are substrates for a number of bacterial nitroreductases and azoreductases, which
376 play key roles in the degradation/detoxification/cometabolism process, suggesting that the
377 environmental pollution could have posed a selective pressure for adaptive rapid evolution of the
378 bacterial enzymes. Alternatively, these enzymes, which are more ancient than their synthetic
379 substrates, may have evolved to reduce as-yet unidentified nitroaromatic natural products. We
380 recent showed that *arsEFG*, widely distributed in anaerobes, confer resistance to trivalent
381 aromatic arsenicals but not to other natural occurring inorganic and organic arsenicals³⁰, which is
382 another example of rapid adaptation to the introduction of anthropogenic organoarsenical. In this
383 study, we demonstrate that *S. meliloti* Rm1021 is capable of reduction of both the nitro and
384 arsenate groups of pentavalent nitroaromatic arsenicals, producing trivalent aminoaromatic
385 arsenicals as final products (Fig. 1 and S4). In analogy with methylarsenicals, pentavalent
386 aromatic arsenicals are much less toxic than inorganic arsenicals, while their trivalent forms are
387 much more toxic than inorganic species²³, suggesting that reduction of aromatic arsenicals is
388 also an activation process. Thus, we propose that *S. meliloti* Rm1021 has gained the capacity to

389 utilize the artificial aromatic arsenicals as an antibiotic to enhance their competitive advantage in
390 contaminated sites – another way of bacterial rapid adaptation to synthetic arsenical
391 contaminants. Trivalent aromatic arsenicals exhibit higher toxicity to bacteria compared to
392 MAs(III)^{23,30,51}, indicating that trivalent aromatic arsenicals would be more potent antibiotics and
393 give a better competitive advantage to the reducers. Similar to the Caco-2 cell⁵², both
394 *Burkholderia* sp. MR1 and *P. putida* KT2440 poorly take up pentavalent aromatic arsenicals (Fig.
395 S3). Given that, one of the critical features that enables *S. meliloti* Rm1021 to reduce
396 pentavalent aromatic arsenicals should be the effective uptake systems, which will be an
397 important future research objective to be elucidated. There are several genes known to confer
398 resistance to trivalent aromatic arsenicals (Fig. 4). As mentioned above, *arsEFG* confers
399 resistance specifically against trivalent nitroaromatic arsenicals via sequential reactions of
400 nitroreduction by ArsEF and extrusion of the resulting amine derivatives by ArsG³⁰. Both the
401 MAs(III) oxidase ArsH and the MAs(III) efflux pump ArsP also detoxify Rox(III), although their
402 catalytic efficiency is relatively lower compared to that for MAs(III)^{23,51}. In contrast, the MAs(III)
403 demethylase Arsl is less selective and effectively degrades both MAs(III) and Rox(III)²⁰. In
404 addition, a recent study shows that *arsK*, the newly identified *ars* gene from *Agrobacterium*
405 *tumefaciens* GW4 that encodes a novel arsenic efflux pump (AtArsK), confers resistance to
406 As(III), antimonite (Sb(III)), MAs(III) and Rox(III)⁵³. Interestingly, *S. meliloti* 1021 carries an *arsRK*
407 operon in one of the two megaplasms pSymB⁵⁴. SmArsK shares 69% identities and 80%
408 positives with AtArsK. SmArsK may function as an efflux pump for secretion of these trivalent

409 organoarsenical antibiotics from *S. meliloti* 1021. In turn, other members of these microbial
410 communities have acquired the resistance mechanisms described above. Given the recent but
411 heavy usage of aromatic arsenicals in animal husbandry as well as the ubiquity of *Sinorhizobium*
412 species in the rhizosphere, our results suggest that agricultural application of aromatic arsenicals
413 fuels bacterial competition for dominance and survival, especially in oxic environments. Microbial
414 breakdown of organoarsenicals can lead to local arsenic contamination, which can alter the soil
415 microbiome and retard plant growth.

416

417

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [xx.xxxx/acs.est.xxxxxxx](https://doi.org/10.1021/acs.est.1c00000). Arsenic biotransformation; oligonucleotide primers used in plasmid constructions; oxidation of trivalent organoarsenicals; chemical structures of arsenicals; bacterial transformation of aromatic arsenicals; time-course analysis of the transformation of nitroaromatic arsenicals by *S. meliloti* Rm1021; nitroreduction of nitroaromatic arsenicals by a *S. meliloti* *ars* operon deletion strain; nitroreduction of Rox(III) by *E. coli* AW3110 cells expressing candidate nitroreductase genes; SmMdaB exhibits NADPH-dependent FAD reductase activity; nitroreduction of Nit(III) *in vivo*; nitroreduction of Nit(V) *in vitro* (PDF)

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Figure legends

Figure 1. Organoarsenical reduction by *S. meliloti* Rm1021. Transformation of the indicated organoarsenicals in the presence or absence of *S. meliloti* Rm1021 and/or *Streptomyces* sp. MD1 was assayed by HPLC-ICP-MS, as described under *Methods and Materials*. (A) Transformation of MAs(V) was analyzed using a C18 reverse-phase column. (B) Transformation of *p*-ASA(V) was analyzed using a C18 reverse-phase column. (C) Transformation of Nit(V) was first analyzed with a C4 reverse-phase column because the retention time of nitroaromatic arsenicals on the C18 column is much longer than the other species. (D) Transformation of Nit(V) was further analyzed using a C18 column because the C4 column cannot separate As(III), *p*-ASA(III) and *p*-ASA(V). (E) Transformation of Rox(V) was first analyzed with a C4 column because the retention time of nitroaromatic arsenicals on the C18 column is much longer than the other species. (F) Transformation of Rox(V) was further analyzed with a C18 column to separate As(III), HAPA(III) and HAPA(V).

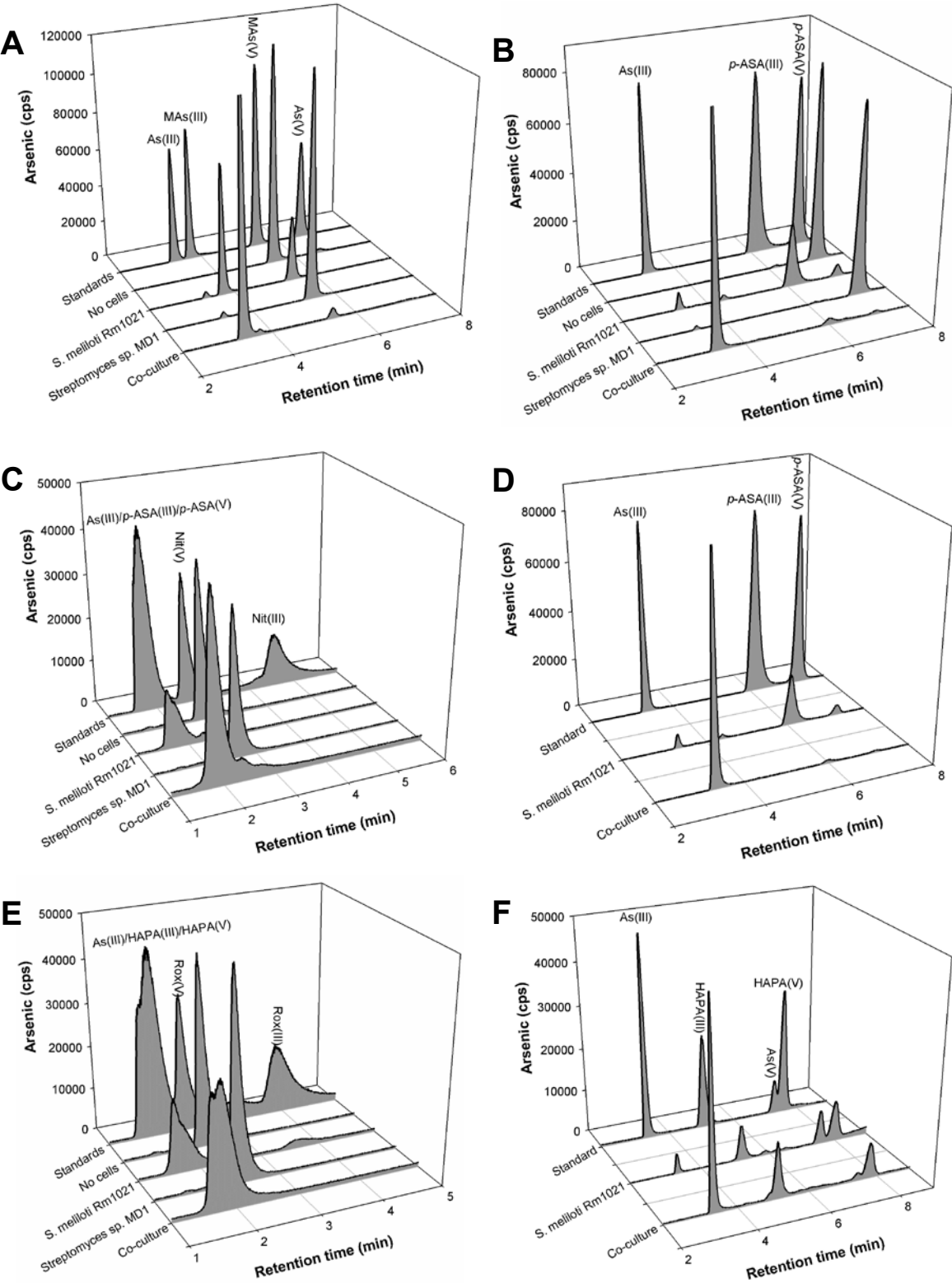
Figure 2. Roxarsone nitroreductase activity of SmMdaB *in vivo*. Nitroreduction of Rox(III) by *SmmdaB*-expressing AW3110 cells was analyzed by HPLC-ICP-MS as described under *Methods and Materials*. As described in the legend to Fig. 1, arsenic species in the culture of AW3110 cells carrying pET28a-*SmmdaB* after the indicated hours was first analyzed by a C4 column (A) and subsequently analyzed with a C18 column (B) to separate As(III), HAPA(III) and HAPA(V). *Vector*, AW3110 cells carrying the empty vector (pET28a); + *SmmdaB*, AW3110 cells carrying pET28a-*SmmdaB*.

Figure 3. Roxarsone nitroreductase activity of SmMdaB *in vitro*. Nitroreduction of Rox(V) by purified SmMdaB was assayed as described under *Methods and Materials*. (A) Effect of GSH

and TCEP on nitroreduction of Rox(V). Nitroreduction of Rox(V) by SmMdaB was carried out with or without 5 mM GSH and/or 1 mM TCEP, and the products were analyzed by HPLC-ICP-MS with a C4 column. (B and C) Nitroreduction of Rox(V) by purified SmMdaB was carried out with 5 mM GSH and 1 mM TCEP and analyzed by HPLC-ICP-MS. As described in the legend to Fig. 1, arsenic species in the reaction mixtures after the indicated time was first analyzed with a C4 reverse-phase column (B) and subsequently analyzed on a C18 column (C) to separate As(III), HAPA(III) and HAPA(V).

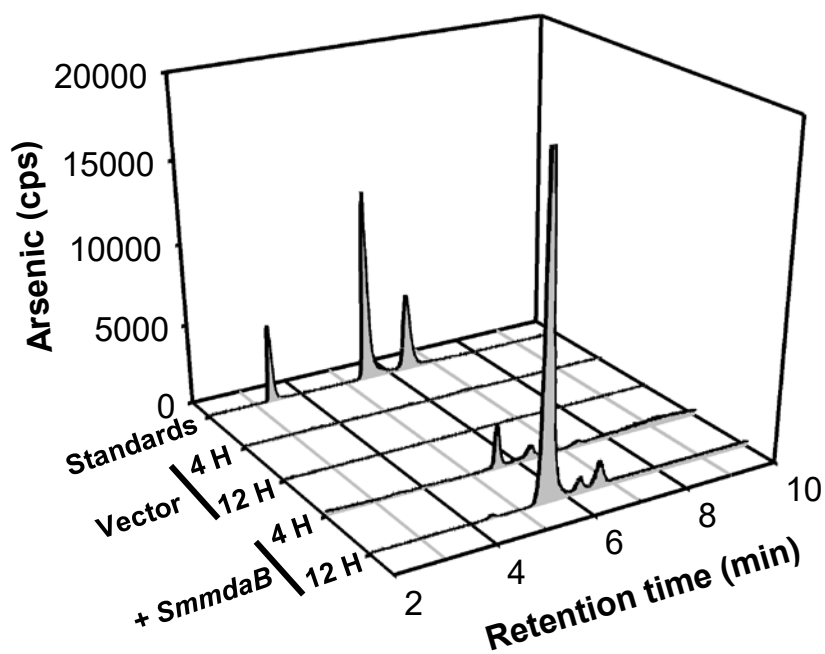
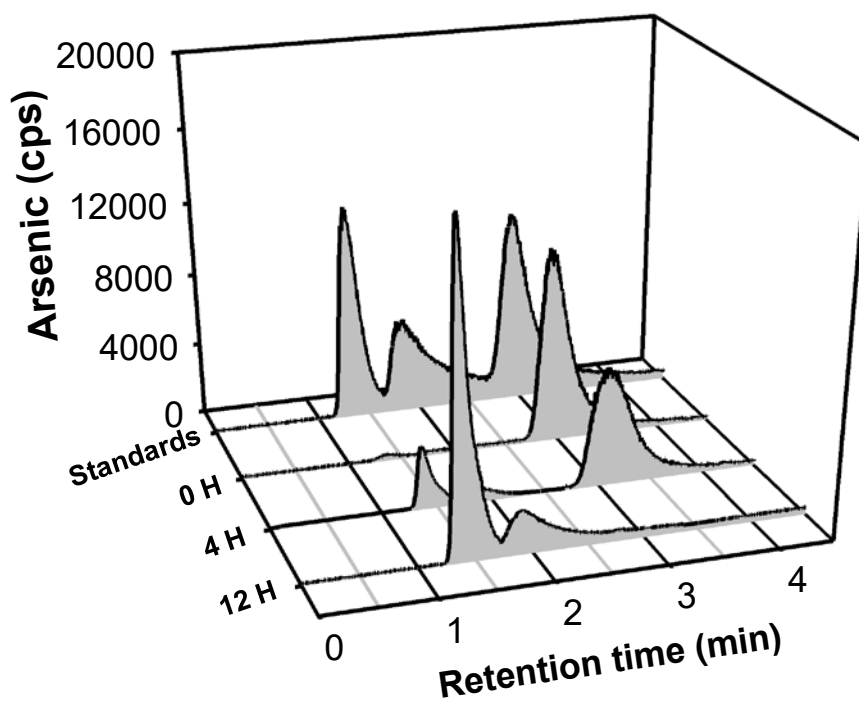
Figure 4. Degradation of pentavalent aromatic arsenicals by an aerobic microbial community is a multistep process. *S. meliloti* takes up pentavalent aromatic arsenicals by unknown transporters, reduces the nitro group by MdaB and the arsenate moiety by unknown reductases. The trivalent aminoaromatic arsenical products are extruded from the cells, possibly by ArsK. The secreted trivalent organoarsenicals act as antibiotics to kill competitors. A small portion of the aromatic arsenicals are degraded into As(III), which flows out of the cell by ArsK or by downhill movement through AqpS⁵². Some community members detoxify the organoarsenical antibiotics by a variety organoarsenical resistance mechanisms, as described in the text. *S. meliloti* degrades the pentavalent aromatic arsenicals by sequential reduction of the nitro and arsenate groups. C-As bond cleavage of the trivalent aminoaromatic arsenicals is carried out by other members of microbial communities such as *Streptomyces* species that carry an *arsI* gene. This is similar to the pathway of reduction of and resistance to the pentavalent methylarsenical herbicide MSMA.

649 Figure 1



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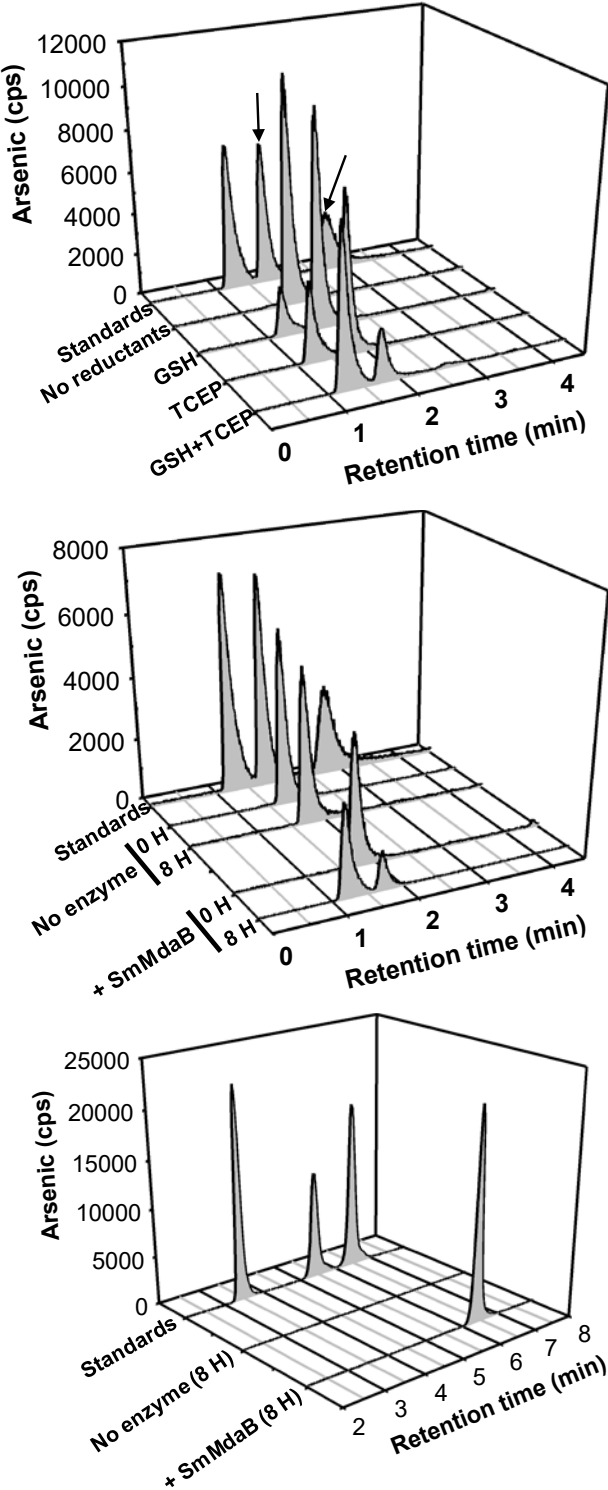
651 Figure 2



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654 Figure 3



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