

1 Submitted to Environmental Science & Technology, Wednesday, July 5, 2019

2 Revised version submitted Friday, October 25, 2019

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

5 Yu Yan^{1,2,†}, Jian Chen^{2,†}, Adriana E. Galván², Luis D. Garbinski², Yong-Guan Zhu^{3,4}, Barry P.

6 Rosen² and Masafumi Yoshinaga^{2*}

7 *¹Department of Environmental Science and Engineering, Huaqiao University, Xiamen 361021,*

8 *China*

9 *²Department of Cellular Biology and Pharmacology, Herbert Wertheim College of Medicine,*

10 *Florida International University, U. S. A.*

11 *³Key Lab of Urban Environment and Health, Institute of Urban Environment, Chinese Academy*
12 *of Sciences, Xiamen 361021, China*

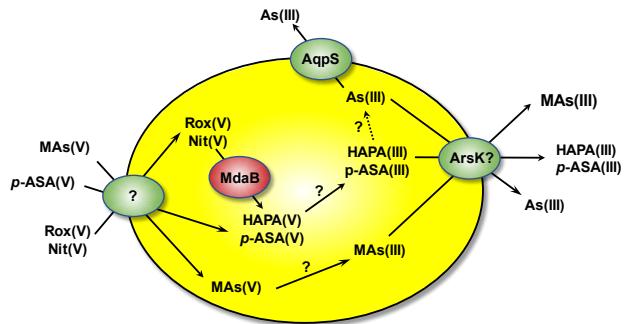
13 *⁴State Key Laboratory of Urban and Regional Ecology, Research Center for Eco-Environmental*
14 *Sciences, Chinese Academy of Sciences, Beijing 100085, China*

15

16 *Correspondence. Masafumi Yoshinaga (myoshina@fiu.edu), Department of Cellular Biology
17 and Pharmacology, Florida International University, Herbert Wertheim College of Medicine,
18 11200 SW 8th Street, AHC1 419G, Miami, Florida, U. S. A. 33199, Tel: (+1) 305-348-1489, Fax:
19 (+1) 305-348-0651

20 #These authors contributed equally

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



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

43

44 **INTRODUCTION**

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

60

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

63 methylarsenicals, both MAs(V) and DMAAs(V), have been utilized as herbicides since the early
64 1970s. Although the use of DMAAs(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), nitarsone
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 promotors is no longer allowed in the United States
77 (<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
78 anthropogenic organoarsenic compounds gradually degrade into more toxic inorganic forms
79 through microbial activities that impact the environment and contaminate our water and food
80 supplies¹⁰⁻¹². A number of bacterial species, both aerobes^{9,13-15} and anaerobes¹⁶⁻¹⁸, have been

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

84

85 We previously identified a microbial community from Florida golf course soil that degrades
86 MAs(V) aerobically¹⁹, where one microbe activates MAs(V) into highly toxic MAs(III) that other
87 member subsequently detoxifies into less toxic inorganic As(III). The MAs(III) demethylation is
88 catalyzed by the Arsl C-As lyase, an Fe(II)-dependent dioxygenase²⁰. Here we demonstrate that
89 pentavalent aromatic arsenicals are also biotransformed into inorganic species via sequential
90 reduction and Arsl-catalyzed C-As bond cleavage by aerobic microbial communities, similar to
91 the pathway of demethylation of the MSMA herbicide. Legume symbiont *Sinorhizobium meliloti*
92 Rm1021 is a typical soil bacterium and forming nitrogen-fixing symbiosis with alfalfa and other
93 legumes. *S. meliloti* Rm1021 has been extensively studied as a model organism for the genetic
94 analysis of symbiotic nitrogen fixation and legume-microbe interaction. The sequencing of the
95 entire genome of *S. meliloti* Rm1021 has been completed in 2001²¹. For As metabolism, *S.*
96 *meliloti* Rm1021 can utilize a unique detoxification pathway wherein reduction of As(V) by
97 arsenate reductase (ArsC) is coupled to downhill transport of As(III) through the
98 aquaglyceroporin (AqpS) channel²². In this study, we demonstrate that the *S. meliloti* Rm1021
99 reduces both MAs(V) and pentavalent aromatic arsenicals, producing trivalent methyl and
100 aromatic arsenicals. Other Rox(V)-degrading aerobes⁹ and anaerobes¹⁶⁻¹⁸ have been reported

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

111

112 **METHODS AND MATERIALS**

113 **Strains and chemicals**

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

120 bacterial strains were cultured aerobically with shaking at 37 °C (*E. coli* strains) or 30 °C (others).
121 Unless otherwise indicated, all chemicals were of analytical or better grade from MilliporeSigma
122 (Burlington, MA). Rox(V) and HAPA(V) were purchased from Thermo Fisher Scientific (Waltham,
123 MA) and Pfaltz & Bauer (Waterbury, CT), respectively. The trivalent organoarsenicals (Rox(III),
124 Nit(III), *p*-ASA(III) and HAPA(III)) were prepared by chemical reduction of their corresponding
125 pentavalent forms as previously described²⁰. In brief, 0.2 mM pentavalent arsenical was mixed
126 with 27 mM Na₂S₂O₃, 66 mM Na₂S₂O₅, and 82 mM H₂SO₄, following which the pH was adjusted
127 to 6 with NaOH. We confirmed by absorption spectroscopy that the chemical reduction method
128 reduces As(V) to As(III) but does not reduce the nitro group of roxarsone and nitarsone. The
129 methylarsonous acid iodide derivative (MAs(III)I₂) synthesized as described²⁸ was used as
130 MAs(III). For confirmation purpose, prepared MAs(III)I₂, *p*-ASA(III) and HAPA(III) were treated
131 with 4.5 % (v/v) H₂O₂ and incubated first for 10 min at room temperature and further for 5 min at
132 80 °C. Oxidized organoarsenicals were analyzed by HPLC-ICP-MS as described below to
133 confirm they match the corresponding pentavalent species (Fig. S1A, C and F). The chemical
134 structures of arsenicals are shown in Fig. S2.

135

136 **Biotransformation of organoarsenicals**

137 *S. meliloti* Rm1021, *S. meliloti* RmP310 (Δ ars), *Burkholderia* sp. MR1 and *P. putida* KT2440 were
138 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 organarsenicals 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

159 \times 2.1 mm; 5 μ m; GL Sciences, Japan) isocratically eluted with a mobile phase consisted with
160 15% acetonitrile (v/v), 5% ethanol (v/v) and 80% water (pH 1.5 adjusted by HCl) (v/v), at a flow
161 rate of 0.3 or 0.6 mL min⁻¹ at 60 °C. For confirmation purpose, samples containing MAs(III),
162 *p*-ASA(III) and HAPA(III) were treated with 4.5 % (v/v) H₂O₂ and incubated first for 10 min at
163 room temperature and further for 5 min at 80 °C. Oxidized samples were analyzed by
164 HPLC-ICP-MS to confirm their species (Fig. S1B, D, E and G). Each amount of the indicated
165 arsenic species in bacterial culture/co-culture samples was quantified from the corresponding
166 peak area using Chromera Chromatography Data System version 2.1 (Perkin Elmer) according
167 to standard curves prepared with standard solutions in the range of 0.5-2 μ M in water. The
168 results from the triplicated independent experiments are summarized in Table S1.

169

170 **Plasmid construction**

171 By conducting a BLAST (Basic Local Alignment Search Tool) search of the genome of *S. meliloti*
172 1021, the genes encoding SmAzoR (NCBI accession No.: NP_385442), SmMdaB (NP_387022),
173 SmMsuE (NP_438025) and SmNemA (NP_385670), SmAZR (NP_386600) and SmNitB
174 (NP_384119) were chosen as candidates responsible for the nitroreduction of roxarsone and
175 nitarsone (See *Results and discussion*). For construction of plasmids for expression of *SmazoR*,
176 *SmnemA* and *SmnitB*, each gene was PCR-amplified from total genomic DNA of *S. meliloti*
177 Rm1021 with *Pfu*Turbo DNA polymerase (Agilent Technologies Inc., Santa Clara, CA) using the

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

184

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

186 *In vivo* nitroreduction of Rox(III) by *E. coli* AW3110(DE3) cells carrying the constructed plasmids
187 was analyzed spectrophotometrically, as described previously³⁰. Briefly, After overnight growth in
188 LB medium supplemented with 25 μ g mL⁻¹ kanamycin and 0.3 mM isopropyl
189 β -D-1-thiogalactopyranoside (IPTG) at 30 °C, the cells were washed once with low phosphate
190 medium³¹ supplemented with 0.2% D-glucose and suspended in glucose-free M9 medium³² at a
191 cell density of $A_{600\text{ nm}} = 10.0$. The cell suspensions were then incubated with 60 μ M Rox(III) at
192 30 °C with shaking (200 rpm) for 6 h. The nitroreductase activity of cells was estimated by loss of
193 absorbance at 410nm determined with a Synergy H4 Hybrid Multi-Mode microplate reader
194 (BioTek Instruments, Inc., Winooski, VT). *In vivo* nitroreductase activity of SmMdaB was also
195 analyzed by arsenic speciation. Cell suspensions of *E. coli* AW3110(DE3) cells harboring
196 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.

199

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 $A_{600\text{ nm}}$ 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 $A_{280\text{ nm}}$ ($\epsilon = 53,910 \text{ M}^{-1}\cdot\text{cm}^{-1}$). SmMdaB-containing fractions
213 were divided into small portions, rapidly frozen, and stored at -80 °C until use.

214

215 **FAD reductase activity**

216 The amount of flavin adenine dinucleotide (FAD) in purified SmMdaB was quantified by
217 absorption at 450 nm using a molar extinction coefficient of $11,300 \text{ M}^{-1} \cdot \text{cm}^{-1}$. Reduction of FAD
218 by SmMdaB was assayed in 25 mM Bis-Tris propane buffer (pH 7.0) containing 1 mM EDTA and
219 0.1 mg mL^{-1} bovine serum albumin (BSA) at 37°C . 0.2 mM NADPH was incubated with 1 μM
220 SmMdaB and/or 25 μM FAD and the oxidation of NADPH was monitored by the decrease in
221 absorbance at 340 nm ($\epsilon = 6,220 \text{ M}^{-1} \cdot \text{cm}^{-1}$).

222

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

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

230

231 **Results and discussion**

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

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

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

238

239 **Reduction of aromatic arsenicals by *S. meliloti***

240 We previously identified the *arsl* gene for MAs(III) demethylation from *Bacillus* sp. MD1, an
241 MAs(III)-demethylating bacterial isolate from Florida golf course soil²⁰. Cells of the
242 arsenic-hypersensitive *Escherichia coli* strain AW3110²⁷ expressing *arsl* degrade trivalent
243 aromatic arsenicals, including *p*-ASA(III), Nit(III) and Rox(III), into inorganic As(III), suggesting
244 that Arsl also catalyzes aerobic breakdown of these antimicrobial animal growth promoters. It
245 seemed reasonable to consider that pentavalent aromatic arsenicals also undergo sequential
246 reduction and Arsl-catalyzed C-As bond cleavage by microbial communities. This is formally
247 similar to demethylation of the MSMA herbicide by the MAs(V)-reducing *Burkholderia* sp. MR1
248 and the MAs(III)-demethylating *Streptomyces* sp. MD1 from Florida golf course soil. However,
249 neither of *Burkholderia* sp. MR1 nor *Pseudomonas putida* KT2440, the other known MAs(V)
250 reducer, are able to reduce pentavalent aromatic arsenicals (Fig. S3). In contrast, *S. meliloti*
251 Rm1021 reduces them to the trivalent species. As with MAs(V), *S. meliloti* Rm1021 converts
252 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

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

275

276 **Identification of the gene for nitroreduction of aromatic arsenicals**

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

290

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

292 Possibilities include known bacterial nitroreductases and azoreductases that have central roles
293 in reduction of nitroaromatic compounds^{40,41}. Although their physiological roles remain unclear,
294 these enzymes have gained considerable attention because of their potential applications in
295 cancer treatment and bioremediation. A number of bacterial nitroreductases and azoreductases
296 have been identified to activate nitroaromatic anticancer prodrugs such as CB1954
297 (5-(aziridine-1-yl)-2,4-dinitrobenzamide) and PR-104A
298 (2-((2-bromoethyl)-2-[(2-hydroxyethyl)amino]carbonyl}-4,6-dinitroanilino)ethyl
299 methanesulfonate)⁴²⁻⁴⁴ and nitroaromatic antimicrobial prodrugs nitrofurans such as
300 nitrofrazone and nitrofurantoin^{45,46} and to degrade nitroaromatic environmental contaminants
301 such as 2,4,6-trinitrotoluene (TNT)⁴⁷⁻⁴⁹. Because these nitroaromatic compounds have
302 similarities with Rox(V) and Nit(V), it was reasonable to consider that some are capable of
303 reduction of Nit(V) and Rox(V). One candidate is ArsH, which physiologically oxidizes MAs(III) to
304 MAs(V) for detoxification²¹. ArsH from *Pseudomonas aeruginosa* PAO1 has been demonstrated
305 to reduce a nitroaromatic antibiotic (nitrofrazone) to the hydroxylamine derivative⁴⁶. However, a
306 *S. meliloti* Rm1021 *ars* operon deletion strain lacking *arsH* (Δ *ars*)^{23,25} retains the ability to reduce
307 nitroaromatic arsenicals (Fig. S5), indicating that little or no contribution of SmArsH. We
308 conducted a BLAST search of the genome of *S. meliloti* 1021 to identify orthologs of AzoR, MdaB,
309 MsuE and NemA enzymes that reduce CB1954 and/or PR-104A to hydroxylamine
310 derivatives⁴²⁻⁴⁴. These include SmAzoR (NCBI accession No.: NP_385442), SmMdaB
311 (NP_387022), SmMsuE (NP_438025) and SmNemA (NP_385670). There are also homologs of

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

314

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

331

332 **SmMdaB is nitroaromatic arsenical nitroreductase**

333 MdaB (modulator of drug activity B) has been shown to be an FAD- and NADPH-dependent
334 quinone reductase with nitroreductase activity with various nitroaromatic compounds^{43,46}.
335 Nitroreduction by the *S. meliloti* ortholog SmMdaB was characterized *in vitro* using purified
336 protein. Purified SmMdaB is yellow and exhibits an absorption spectrum with λ_{max} at 450 nm due
337 to 61.2 % FAD occupancy. However, it still requires supplementation with FAD in addition to
338 NADPH to exhibit FAD reductase activity (Fig. S7). SmMdaB requires an additional reducing
339 potential such as GSH or TCEP for nitroreductase activity with Rox(V) in addition to FAD and
340 NADPH (Fig. 3A). The added reductant may be required to reduce the nitro group all the way to
341 the amine. In contrast, purified MdaB from *P. aeruginosa* PAO1 (PaMdaB) catalyzes
342 nitroreduction of nitrofranzone with only NADPH as reductant *in vitro*, but the product is the
343 hydroxylamine derivative, not the amine⁴⁶. No nitroreduction was observed without enzyme (Fig.
344 3B). SmMdaB also reduces nitarsone to *p*-ASA both *in vivo* (Fig. S8) and *in vitro* (Fig. S9).
345 Altogether, our results clearly demonstrate that SmMdaB is nitroarmoatic arsenical
346 nitroreductase.

347

348 Since *S. meliloti* Rm1021 first reduces the nitro group of Rox(V) and Nit(V) to form HAPA(V) and
349 *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 nitarsone, 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 ArsI 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 megaplasmids 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

418 **Supporting Information**

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

427 **Acknowledgments**

428 This work was supported by NSF BIO/MCB Grant 1817962 and a pilot project grant from the
429 Herbert Wertheim College of Medicine (Project #800008403) to MY, NIH R01 grants GM55425 to
430 BPR, grants from the National Natural Science Foundation of China (41807410), the Scientific
431 Research Funds of Huaqiao University (Z18Y0021) and the Chinese Scholarship Council to YY
432 and the National Key R&D Program of China (2017YFE0107300) to YGZ.

433

434

435

436

437

438 **AUTHOR CONTRIBUTIONS:** Y.Y., J.C., A.E.G., and L.D.G. conducted the experiments. Y.Y.,
439 J.C., B.P.R. and M.Y. designed the experiments and wrote the manuscript. Y.G.Z. provided
440 advice and suggestions. The authors declare no competing financial interest.

441

442 **REFERENCES**

443 1. Zhu, Y. G.; Xue, X. M.; Kappler, A.; Rosen, B. P.; Meharg, A. A. Linking genes to microbial
444 biogeochemical cycling: lessons from arsenic. *Environ. Sci. Technol.* **2017**, *51* (13), 7326–7339.

445 2. Zhu, Y. G.; Yoshinaga, M.; Zhao, F. J.; Rosen, B. P. Earth abides arsenic biotransformations.
446 *Annu. Rev. Earth Planet. Sci.* **2014**, *42* (1), 443–467.

447 3. Qin, J.; Rosen, B. P.; Zhang, Y.; Wang, G.; Franke, S.; Rensing, C. Arsenic detoxification and
448 evolution of trimethylarsine gas by a microbial arsenite s-adenosylmethionine methyltransferase.
449 *Proc. Natl. Acad. Sci. U. S. A.* **2006**, *103* (7), 2075–2080.

450 4. Li, J.; Pawitwar, S. S.; Rosen, B. P. The organoarsenical biocycle and the primordial antibiotic
451 methylarsenite. *Metallomics* **2016**, *8* (10), 1047–1055.

452 5. Chen, S. C.; Sun, G. X.; Rosen, B. P.; Zhang, S. Y.; Deng, Y.; Zhu, B. K.; Rensing, C.; Zhu, Y.
453 G. Recurrent horizontal transfer of arsenite methyltransferase genes facilitated adaptation of life
454 to arsenic. *Sci. Rep.* **2017**, *7* (1), 7741.

455 6. Chen, J.; Yoshinaga, M.; Rosen, B. P. The antibiotic action of methylarsenite is an emergent
456 property of microbial communities. *Mol. Microbiol.* **2019**, *111* (2), 487–494.

457 7. Gannon, T. W.; Polizzotto, M. L. MSMA: knowledge gaps to aid appropriate regulation of an
458 efficacious herbicide. *Agric. Environ. Lett.* **2016**, *1* (160025), 1–3.

459 8. Garbarino, J. R.; Bednar, A. J.; Rutherford, D. W.; Beyer, R. S.; Wershaw, R. L. Environmental
460 fate of roxarsone in poultry litter. I. Degradation of roxarsone during composting. *Environ. Sci.
461 Technol.* **2003**, *37* (8), 1509–1514.

462 9. Huang, K.; Peng, H.; Gao, F.; Liu, Q. Q.; Lu, X.; Shen, Q.; Le, X. C.; Zhao, F. J.
463 Biotransformation of arsenic-containing roxarsone by an aerobic soil bacterium *Enterobacter* sp.
464 Cz-1. *Environ. Pollut.* **2019**, *247*, 482–487.

465 10. Feng, M.; Schrlau, J. E.; Snyder, R.; Snyder, G. H.; Chen, M.; Cisar, J. L.; Cai, Y. Arsenic
466 transport and transformation associated with MSMA application on a golf course green. *J. Agric.
467 Food Chem.* **2005**, *53* (9), 3556–3562.

468 11. Yao, L.; Huang, L.; He, Z.; Zhou, C.; Lu, W.; Bai, C. Delivery of roxarsone via chicken
469 diet → chicken → chicken manure → soil → rice plant. *Sci. Total Environ.* **2016**, *566–567*,
470 1152–1158.

471 12. D'Angelo, E.; Zeigler, G.; Beck, E. G.; Grove, J.; Sikora, F. Arsenic species in broiler (*Gallus*
472 *gallus domesticus*) litter, soils, maize (*Zea mays L.*), and groundwater from litter-amended fields.
473 *Sci. Total Environ.* **2012**, *438*, 286–292.

474 13. Maki, T.; Takeda, N.; Hasegawa, H.; Ueda, K. Isolation of monomethylarsonic

475 acid-mineralizing bacteria from arsenic contaminated soils of Ohkunoshima island. *Appl.*

476 *Organomet. Chem.* **2006**, 20 (9), 538–544.

477 14. Lehr, C. R.; Polishchuk, E.; Radoja, U.; Cullen, W. R. Demethylation of methylarsenic

478 species by *Mycobacterium neoaurum*. *Appl. Organomet. Chem.* **2003**, 17 (11), 831–834.

479 15. Quinn, J. P.; McMullan, G. Carbon-arsenic bond cleavage by a newly isolated gram-negative

480 bacterium, strain ASV2. *Microbiology* **1995**, 141 (Pt 3, 721–725.

481 16. Fisher, E.; Dawson, A. M.; Polshyna, G.; Lisak, J.; Crable, B.; Perera, E.; Ranganathan, M.;

482 Thangavelu, M.; Basu, P.; Stolz, J. F. Transformation of inorganic and organic arsenic by

483 *Alkaliphilus Oremlandii* sp. nov. strain OhLLAs. *Ann. N. Y. Acad. Sci.* **2008**, 1125, 230–241.

484 17. Han, J. C.; Zhang, F.; Cheng, L.; Mu, Y.; Liu, D. F.; Li, W. W.; Yu, H. Q. Rapid release of

485 arsenite from roxarsone bioreduction by Exoelectrogenic bacteria. *Environ. Sci. Technol. Lett.*

486 **2017**, 4 (8), 350–355.

487 18. Chen, J.; Rosen, B. P. Organoarsenical biotransformations by *Shewanella putrefaciens*.

488 *Environ. Sci. Technol.* **2016**, 50 (15), 7956–7963.

489 19. Yoshinaga, M.; Cai, Y.; Rosen, B. P. Demethylation of methylarsonic acid by a microbial

490 community. *Environ. Microbiol.* **2011**, 13 (5), 1205–1215.

491 20. Yoshinaga, M.; Rosen, B. P. A C-As lyase for degradation of environmental organoarsenical

492 herbicides and animal husbandry growth promoters. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, 111

493 (21), 7701–7706.

494 21. Yang, H. C.; Cheng, J.; Finan, T. M.; Rosen, B. P.; Bhattacharjee, H. Novel pathway for
495 arsenic detoxification in the legume symbiont *Sinorhizobium meliloti*. *J. Bacteriol.* **2005**, *187* (20),
496 6991–6997.

497 22. Galibert, F.; Finan, T.M.; Long, S.R.; Puhler, A.; Abola, P.; Ampe, F.; Barloy-Hubler, F.;
498 Barnett, M.J.; Becker, A.; Boistard, P.; Bothe, G.; Bouthy, M.; Bowser, L.; Buhrmester, J.;
499 Cadieu, E.; Capela, D.; Chain, P.; Cowie, A.; Davis, R.W.; Dreano, S.; Federspiel, N.A.; Fisher,
500 R.F.; Gloux, S.; Godrie, T.; Goffeau, A.; Golding, B.; Gouzy, J.; Gurjal, M.; Hernandez-Lucas, I.;
501 Hong, A.; Huizar, L.; Hyman, R.W.; Jones, T.; Kahn, D.; Kahn, M.L.; Kalman, S.; Keating, D.H.;
502 Kiss, E.; Komp, C.; Lelaure, V.; Masuy, D.; Palm, C.; Peck, M.C.; Pohl, T.M.; Portetelle, D.;
503 Purnelle, B.; Ramsperger, U.; Surzycki, R.; Thebault, P.; Vandenbol, M.; Vorholter, F.J.; Weidner,
504 S.; Wells, D.H.; Wong, K.; Yeh, K.C.; Batut, J. The composite genome of the legume symbiont
505 *Sinorhizobium meliloti*. *Science* **2001**, *293* (5530), 668-672.

506 23. Chen, J.; Bhattacharjee, H.; Rosen, B. P. ArsH is an organoarsenical oxidase that confers
507 resistance to trivalent forms of the herbicide monosodium methylarsenate and the poultry growth
508 promoter roxarsone. *Mol. Microbiol.* **2015**, *96* (5), 1042–1052.

509 24. Meade, H. M.; Long, S. R.; Ruvkun, G. B.; Brown, S. E.; Ausubel, F. M. Physical and genetic
510 characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by
511 transposon Tn5 mutagenesis. *J. Bacteriol.* **1982**, *149* (1), 114–122.

512 25. Yang, H. C. Functional and structural studies of a novel arsenic detoxification pathway in the
513 legume symbiont *Sinorhizobium meliloti*, Wayne State University School of Medicine, 2008.

514 26. Bagdasarian, M.; Lurz, R.; Rückert, B.; Franklin, F. C. H.; Bagdasarian, M. M.; Frey, J.;
515 Timmis, K. N. Specific-purpose plasmid cloning vectors II. Broad host range, high copy number,
516 RSF 1010-derived vectors, and a host-vector system for gene cloning in *Pseudomonas*. *Gene*
517 **1981**, *16* (1–3), 237–247.

518 27. Carlin, A.; Shi, W.; Dey, S.; Rosen, B. P. The *ars* operon of *Escherichia coli* confers arsenical
519 and antimonal resistance. *J. Bacteriol.* **1995**, *177* (4), 981–986.

520 28. Stice, S.; Liu, G.; Matulis, S.; Boise, L. H.; Cai, Y. Determination of multiple human arsenic
521 metabolites employing high performance liquid chromatography inductively coupled plasma
522 mass spectrometry. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2016**, *1009*, 55–65

523 29. Maki, T.; Hasegawa, H.; Watarai, H.; Ueda, K. Classification for
524 dimethylarsenate-decomposing bacteria using a restrict fragment length polymorphism analysis
525 of 16s rRNA genes. *Anal. Sci.* **2004**, *20* (1), 61–68.

526 30. Chen, J.; Zhang, J.; Rosen, B. P. Role of ArsEFG in Roxarsone and Nitarsone Detoxification
527 and Resistance. **2019**, *53* (11), 6182–6191.

528 31. Oden, K. L.; Gladysheva, T. B.; Rosen, B. P. Arsenate reduction mediated by the
529 plasmid-encoded ArsC protein is coupled to glutathione. *Mol. Microbiol.* **1994**, *12* (2), 301–306.

530 32. Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular cloning: a laboratory manual*, 2nd ed.;

531 Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, USA, 1989.

532 33. Parker, G. F.; Higgins, T. P.; Hawkes, T.; Robson, R. L. *Rhizobium (Sinorhizobium) meliloti*

533 *phn* genes: Characterization and identification of their protein products. *J. Bacteriol.* **1999**, *181*

534 (2), 389–395.

535 34. Galleguillos, C.; Aguirre, C.; Miguel Barea, J.; Azcón, R. Growth promoting effect of two

536 *Sinorhizobium meliloti* strains (a wild type and its genetically modified derivative) on a

537 non-legume plant species in specific interaction with two arbuscular mycorrhizal fungi. *Plant Sci.*

538 **2000**, *159* (1), 57–63.

539 35. Nadar, V. S.; Yoshinaga, M.; Pawitwar, S. S.; Kandavelu, P.; Sankaran, B.; Rosen, B. P.

540 Structure of the Arsl C-As lyase: insights into the mechanism of degradation of organoarsenical

541 herbicides and growth promoters. *J. Mol. Biol.* **2016**, *428* (11), 2462–2473.

542 36. Yan, Y.; Ye, J.; Xue, X. M.; Zhu, Y. G. Arsenic demethylation by a C-As lyase in

543 cyanobacterium *Nostoc* sp. PCC 7120. *Environ. Sci. Technol.* **2015**, *49* (24), 14350–14358.

544 37. Cortinas, I.; Field, J. a; Kopplin, M.; Garbarino, J. R.; Gandolfi, A. J.; Sierra-Alvarez, R.

545 Anaerobic biotransformation of roxarsone and related N-substituted phenylarsonic acids.

546 *Environ. Sci. Technol.* **2006**, *40* (9), 2951–2957.

547 38. Stolz, J. F.; Perera, E.; Kilonzo, B.; Kail, B.; Crable, B.; Fisher, E.; Ranganathan, M.; Wormer,

548 L.; Basu, P. Biotransformation of 3-Nitro-4-Hydroxybenzene Arsonic Acid (Roxarsone) and
549 release of inorganic arsenic by *Clostridium* species. *Environ. Sci. Technol.* **2007**, *41* (3),
550 818–823.

551 39. Yang, Y.; Chen, J.; Qiu, D.; Zhou, J. Roles of UndA and MtrC of *Shewanella putrefaciens*
552 W3-18-1 in iron reduction. *BMC Microbiol.* **2013**, *13* (1), 267.

553 40. Roldán, M. D.; Pérez-Reinado, E.; Castillo, F.; Moreno-Vivián, C. Reduction of
554 polynitroaromatic compounds: the bacterial nitroreductases. *FEMS Microbiol. Rev.* **2008**, *32* (3),
555 474–500.

556 41. Suzuki, H. Remarkable diversification of bacterial azoreductases: primary sequences,
557 structures, substrates, physiological roles, and biotechnological applications. *Appl. Microbiol.*
558 *Biotechnol.* **2019**, 3965–3978.

559 42. Prosser, G. A.; Copp, J. N.; Syddall, S. P.; Williams, E. M.; Smaill, J. B.; Wilson, W. R.;
560 Patterson, A. V.; Ackerley, D. F. Discovery and evaluation of *Escherichia coli* nitroreductases
561 that activate the anti-cancer Prodrug CB1954. *Biochem. Pharmacol.* **2010**, *79* (5), 678–687.

562 43. Prosser, G. A.; Copp, J. N.; Mowday, A. M.; Guise, C. P.; Syddall, S. P.; Williams, E. M.;
563 Horvat, C. N.; Swe, P. M.; Ashoorzadeh, A.; Denny, W. A.; Smaill, J. B.; Patterson, A. V.;
564 Ackerley, D. F. Creation and screening of a multi-family bacterial oxidoreductase library to
565 discover novel nitroreductases that efficiently activate the bioreductive prodrugs CB1954 and
566 PR-104A. *Biochem. Pharmacol.* **2013**, *85* (8), 1091–1103.

567 44. Green, L. k.; Storey, M. A.; Williams, E. M.; Patterson, A. V.; Smaill, J. B.; Copp, J. N.;

568 Ackerley, D. F. The flavin reductase MsuE is a novel nitroreductase that can efficiently activate

569 two promising next-generation prodrugs for gene-directed enzyme prodrug therapy. *Cancers*

570 (*Basel*). **2013**, *5* (3), 985–997.

571 45. Race, P. R.; Lovering, A. L.; Green, R. M.; Ossor, A.; White, S. A.; Searle, P. F.; Wrighton, C.

572 J.; Hyde, E. I. Structural and mechanistic studies of *Escherichia coli* nitroreductase with the

573 antibiotic nitrofurazone. reversed binding orientations in different redox states of the enzyme. *J.*

574 *Biol. Chem.* **2005**, *280* (14), 13256–13264.

575 46. Crescente, V.; Holland, S. M.; Kashyap, S.; Polycarpou, E.; Sim, E.; Ryan, A. Identification of

576 novel members of the bacterial azoreductase family in *Pseudomonas aeruginosa*. *Biochem. J.*

577 **2016**, *473* (5), 549–558.

578 47. González-Pérez, M. M.; Van Dillewijn, P.; Wittich, R. M.; Ramos, J. L. *Escherichia coli* has

579 multiple enzymes that attack TNT and release nitrogen for growth. *Environ. Microbiol.* **2007**, *9* (6),

580 1535–1540.

581 48. Liu, G.; Zhou, J.; Lv, H.; Xiang, X.; Wang, J.; Zhou, M.; Qv, Y. Azoreductase from

582 *Rhodobacter sphaeroides* AS1.1737 is a flavodoxin that also functions as nitroreductase and

583 flavin mononucleotide reductase. *Appl. Microbiol. Biotechnol.* **2007**, *76* (6), 1271–1279.

584 49. Kutty, R.; Bennett, G. N. Biochemical characterization of trinitrotoluene transforming

585 oxygen-insensitive nitroreductases from *Clostridium acetobutylicum* ATCC 824. *Arch. Microbiol.*

586 2005, 184 (3), 158–167.

587 50. Peng, H.; Hu, B.; Liu, Q.; Li, J.; Li, X. F.; Zhang, H.; Le, X. C. Methylated phenylarsenical
588 metabolites discovered in chicken liver. *Angew. Chemie Int. Ed.* **2017**, 1–6.

589 51. Chen, J.; Madegowda, M.; Bhattacharjee, H.; Rosen, B. P. ArsP: A methylarsenite efflux
590 permease. *Mol. Microbiol.* **2015**, 98 (4), 625–635.

591 52. Liu, Q.; Leslie, E.M.; Le, X.C. Accumulation and transport of Roxarsone, Arsenobetaine, and
592 inorganic arsenic using the human immortalized Caco-2 cell line. *J Agr. Food Chem.* **2016**, 64
593 (46), 8902–8908.

594 53. Shi, K.; Li, C.; Rensing, C.; Dai, X.; Fan, X.; Wang, G. Efflux transporter ArsK is responsible
595 for bacterial resistance to arsenite, antimonite, trivalent roxarsone, and methylarsenite. *Appl.*
596 *Environ. Microbiol.* **2018**, 84 (24), e01842-18.

597 54. Finan, T. M.; Weidner, S.; Wong, K.; Buhrmester, J.; Chain, P.; Vorholter, F. J.;
598 Hernandez-Lucas, I.; Becker, A.; Cowie, A.; Gouzy, J.; Golding, B.; Pühler, A. The complete
599 sequence of the 1,683-Kb PSymB megaplasmid from the N₂-fixing endosymbiont *Sinorhizobium*
600 *meliloti*. *Proc. Natl. Acad. Sci.* **2001**, 98 (17), 9889–9894.

601

602

603 **Figure legends**

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

616

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

624

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

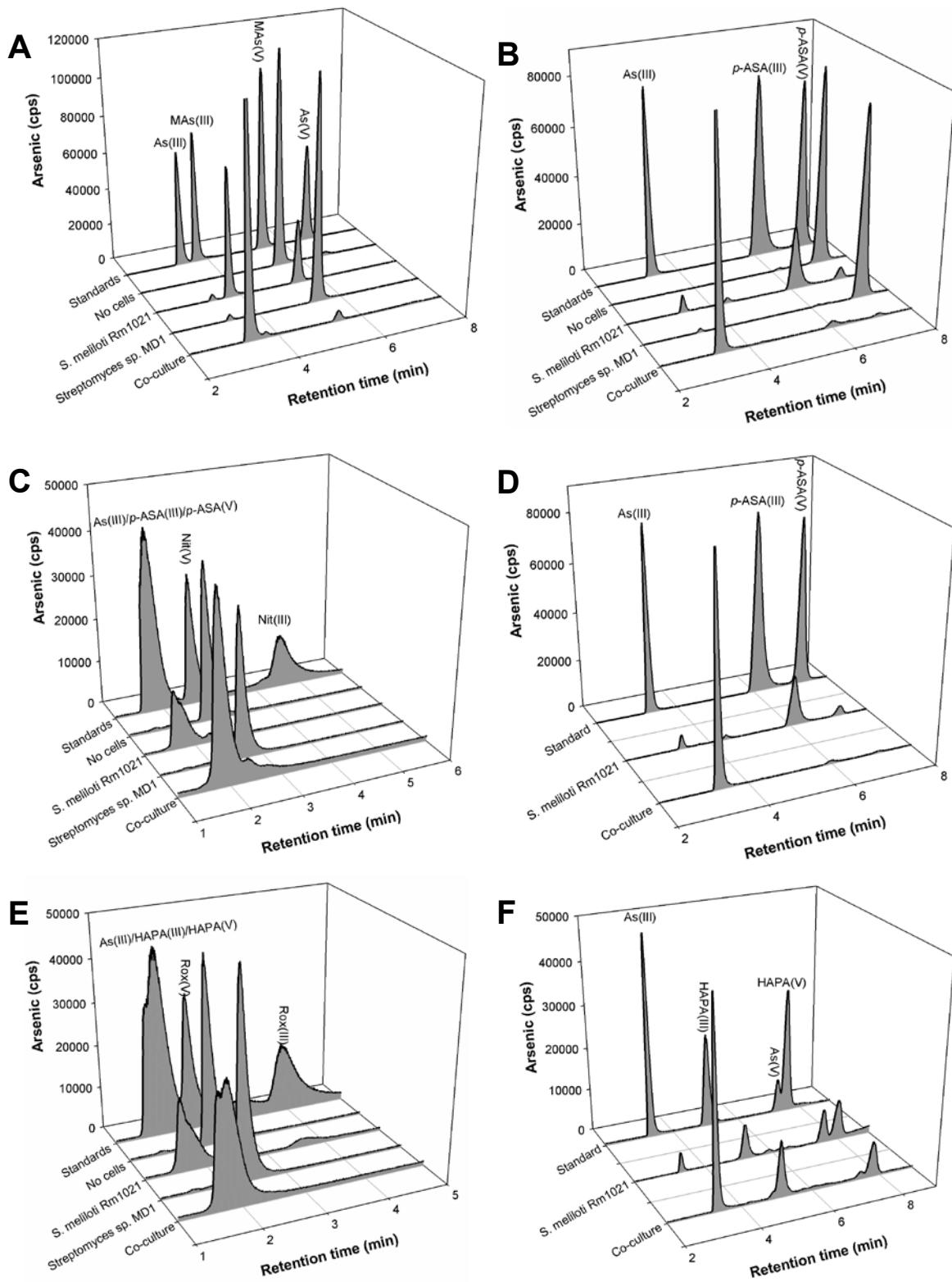
627 and TCEP on nitroreduction of Rox(V). Nitroreduction of Rox(V) by SmMdaB was carried out
628 with or without 5 mM GSH and/or 1 mM TCEP, and the products were analyzed by
629 HPLC-ICP-MS with a C4 column. (B and C) Nitroreduction of Rox(V) by purified SmMdaB was
630 carried out with 5 mM GSH and 1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

634

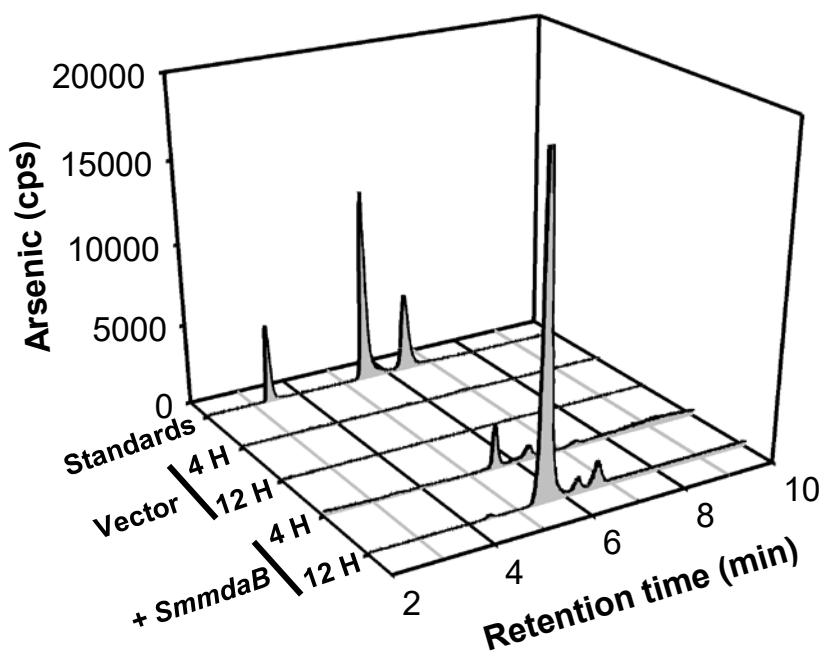
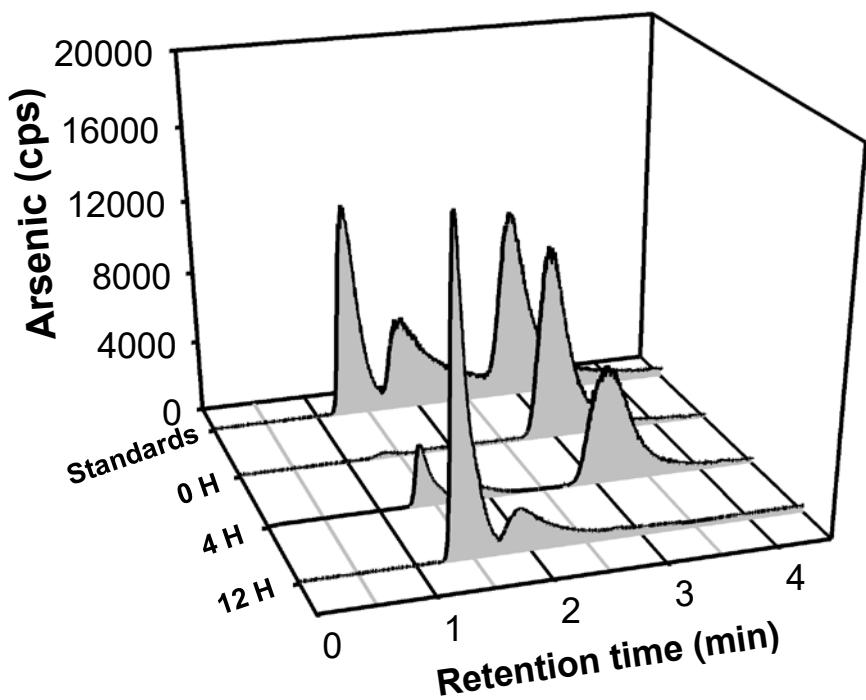
635 **Figure 4. Degradation of pentavalent aromatic arsenicals by an aerobic microbial**
636 **community is a multistep process.** *S. meliloti* takes up pentavalent aromatic arsenicals by
637 unknown transporters, reduces the nitro group by MdaB and the arsenate moiety by unknown
638 reductases. The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

648

649 Figure 1



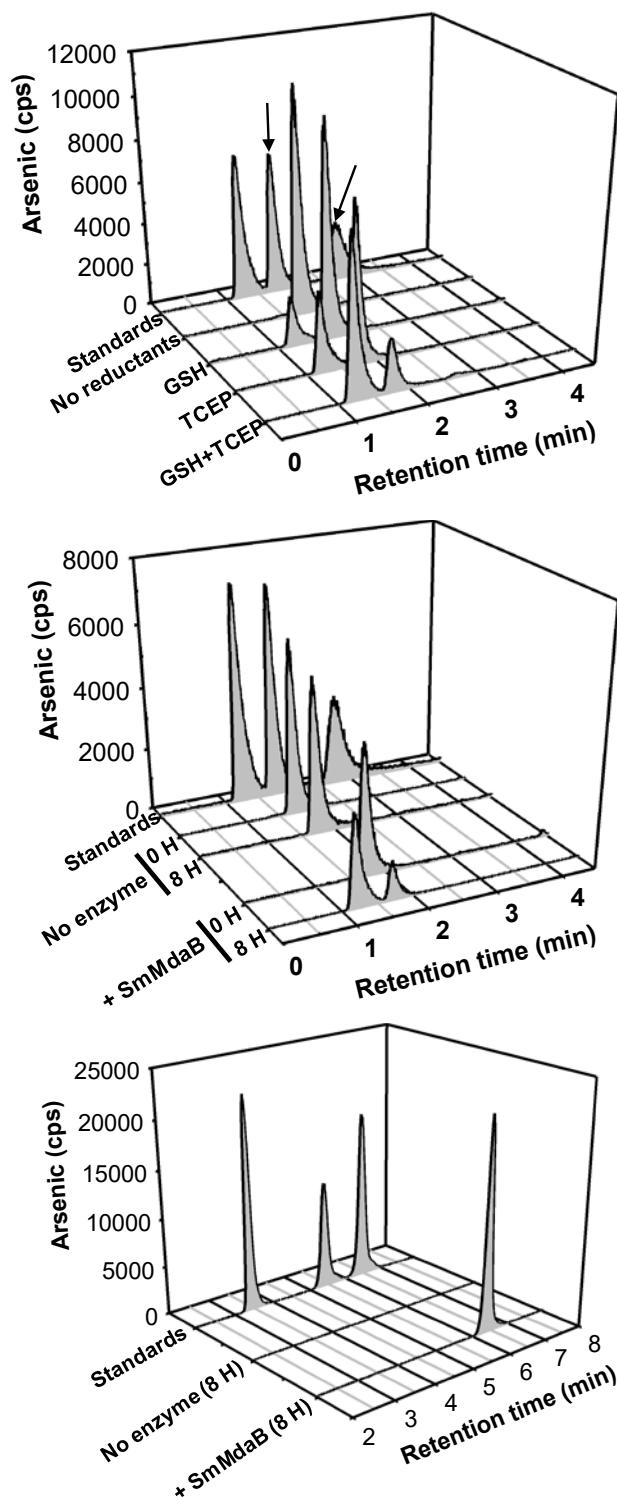
651 Figure 2



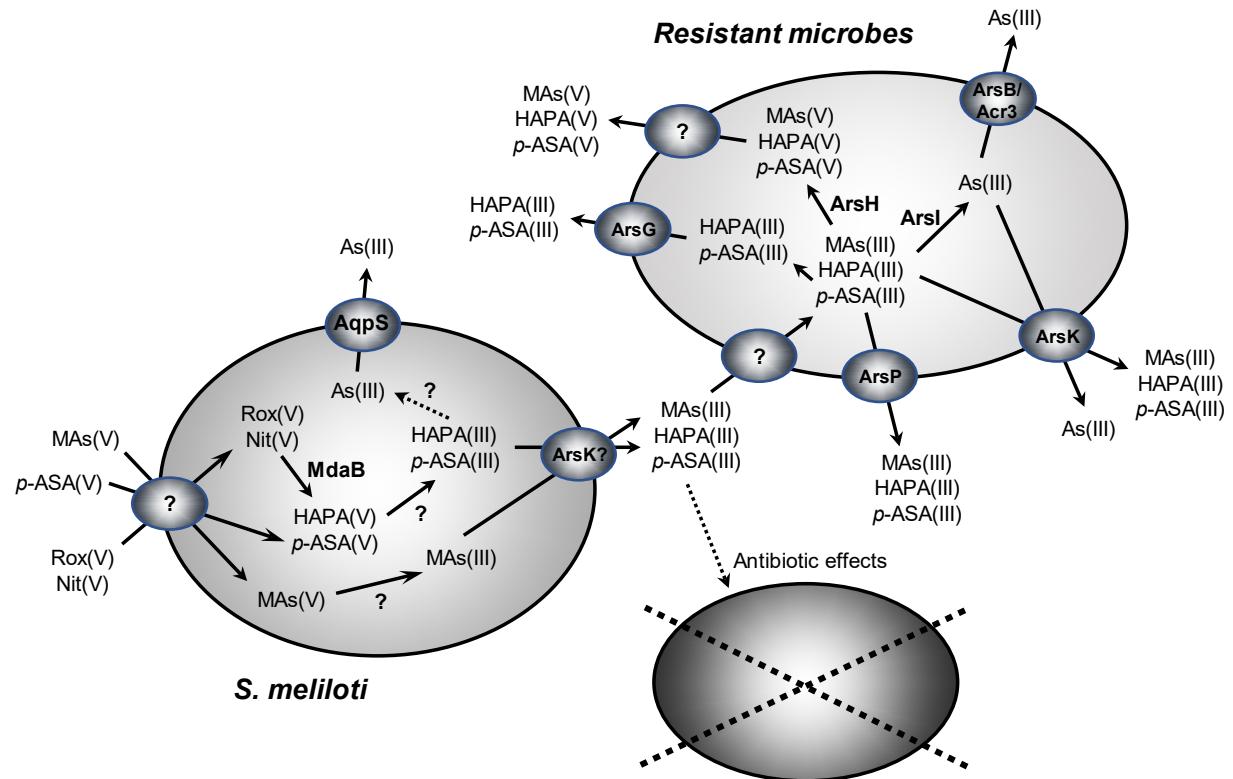
652

653

654 Figure 3



657 Figure 4



658