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ArsH is an organoarsenical oxidase that confers resistance to trivalent forms of the herbicide MSMA and the poultry growth promoter roxarsone

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Abstract

Environmental organoarsenicals are produced by microorganisms and are introduced anthropogenically as herbicides and antimicrobial growth promoters for poultry and swine. Nearly every prokaryote has an *ars* (arsenic resistance) operon, and some have an *arsH* gene encoding an atypical flavodoxin. The role of ArsH in arsenic resistance has been unclear. Here we demonstrate that ArsH is an organoarsenical oxidase that detoxifies trivalent methylated and aromatic arsenicals by oxidation to pentavalent species. *Escherichia coli*, which does not have an *arsH* gene, is very sensitive to the trivalent forms of the herbicide monosodium methylarsenate (MSMA or MAs(V)) and antimicrobial growth promoter roxarsone (Rox(V)), as well as to phenylarsenite (PhAs(III), also called phenylarsine oxide or PAO). *Pseudomonas putida* has two chromosomally-encoded *arsH* genes and is highly resistant to the trivalent forms of these organoarsenicals. A derivative of *P. putida* with both *arsH* genes deleted is sensitive to MAs(III), PhAs(III) or Rox(III). *P. putida arsH* expressed in *E. coli* conferred resistance to each trivalent organoarsenical. Cells expressing PpArsH oxidized the trivalent organoarsenicals. PpArsH was purified, and the enzyme *in vitro* similarly oxidized the trivalent organoarsenicals. These results suggest that ArsH catalyzes a novel biotransformation that confers resistance to environmental methylated and aromatic arsenicals.

Introduction

The metalloid arsenic is the most prevalent environmental toxin (Zhu *et al.*, 2014). This Group 1 human carcinogen ranks first on the Agency for Toxic Substances and Disease Registry (ATSDR) Priority List of Hazardous Substances (<http://www.atsdr.cdc.gov/SPL/index.html>). The bioavailability and toxicological properties of arsenic are highly dependant on its chemical form and oxidation state, which are dictated by microbial arsenic biotransformations. Trivalent arsenicals are more toxic than the pentavalent species. There is no single target of trivalent arsenicals, which react with thiol groups in proteins to inhibit numerous biochemical pathways and with small molecule thiols such as reduced glutathione, which results in formation of reactive oxygen species (Jomova *et al.*, 2011). It seems likely that life evolved in an anoxic environment rich in trivalent inorganic As(III).

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Consequently, early organisms must have developed arsenic resistance pathways, and nearly every extant organism has one or more arsenic detoxification pathways (Zhu *et al.*, 2014). In bacteria these are organized in arsenic resistance (*ars*) operons. At a minimum, cells have an *arsR* gene for an As(III)-responsive transcriptional repressor that controls expression of the operon, a gene, either *arsB* or *acr3*, that encodes an As(III) efflux permease, and *arsC* gene for reduction of inorganic arsenate to As(III) (Yang *et al.*, 2012). As described below, there are additional *ars* genes for arsenical biotransformations, all of which are transcriptionally controlled by *arsR*.

The global arsenic biogeocycle is created by the intersection of redox and methylation cycles. The redox state of inorganic arsenic cycles between the relatively innocuous pentavalent arsenate and the considerably more toxic and carcinogenic trivalent arsenite (Stolz *et al.*, 2006). Other microbes have cycles of methylation and demethylation. Cells with an *arsM* gene encoding an As(III) S-adenosylmethionine methyltransferase methylate As(III) to mono-, di- and trimethylated species (Qin *et al.*, 2009, Qin *et al.*, 2006). Trivalent MAs(III) and DMAs(III) are more toxic and carcinogenic than inorganic As(III) and may account for the majority of arsenic-related disease (Engstrom *et al.*, 2011, Thomas & Rosen, 2013). Other microbes have an *arsI* gene encoding a C-As lyase and transform organoarsenicals back to As(III) (Yoshinaga & Rosen, 2014).

A third cycle of arsenic biotransformations has only recently been recognized: the organoarsenical redox cycle. Pentavalent herbicides and antimicrobial growth promoters are in themselves not especially toxic but are transformed into their active forms by microbial reduction (Chen *et al.*, 2014). Organoarsenical reduction takes place environmentally by microbial communities (Yoshinaga *et al.*, 2011), but the pathways of reduction have not been characterized at the molecular level. *ArsH* was first identified as a gene of unknown function in the *ars* operon of *Yersinia* species (Neyt *et al.*, 1997). The presence of an *arsH* gene was reported to provide a slight increase in resistance to both inorganic arsenate and arsenite in *Yersinia* (Neyt *et al.*, 1997) and *Serratia marcescens* (Ryan & Colleran, 2002). In contrast, neither mutation nor overexpression of *arsH* in *Thiobacillus ferrooxidans* (Butcher *et al.*, 2000) and the cyanobacterium *Synechocystis* sp. strain PCC 6803 (Lopez-Maury *et al.*, 2003) affected resistance to inorganic arsenic. We similarly observed that deletion of *arsH* from *Sinorhizobium meliloti* had a modest effect on resistance to both arsenate and arsenite (Yang *et al.*, 2005). There are currently nearly 9000 *ArsH*-related sequences in the NCBI database. Most are found in bacterial, primarily gammaproteobacteria, but there are closely related sequences in members of every kingdom including fungi such as *Aspergillus fumigatus* (Accession # XP_753154; 58% identity, 73% positives), archaea such as *Halogramma salarium* (Accession # WP_009367405; 28% identity, 49% positives), plants such as the eukaryotic green alga *Coccomyxa subellipsoidea* C-169 (Accession # EIE24473; 68% identity, 79% positives) and animals such as the Yangtze River freshwater dolphin *Lipotes vexillifer* (Accession # XP_007470985; 80% identity, 90% positives) and domestic cow *Bos taurus* DAA33786 (71% identity, 80% positives). *ArsH* does not appear to be widely distributed in animals, so the significance of the few deposited mammalian sequences is unclear.

The *ars* operon of *S. meliloti* has four genes: *arsR*, *aqpS* (encoding an As(III)-conducting aquaglyceroporin), *arsC* and *arsH* (Yang *et al.*, 2005). ArsH is an NADPH-dependent FMN reductase that reduces azo dyes and O₂ to H₂O₂ (Ye *et al.*, 2007), and *Synechocystis* ArsH reduces metals such as chromate and ferric iron (Xue *et al.*, 2014). The kinetics of *Synechocystis* ArsH as a quinone reductase has been studied in detail, but no arsenic-related activity was observed (Hervas *et al.*, 2012). ArsH purifies as a tetramer, and the crystal structure of *S. meliloti* ArsH shows that ArsH is a dimer of dimers (Ye *et al.*, 2007). However, no arsenic-related activity was observed with the purified protein. That fact, coupled with the very modest observed increase in resistance to both oxidized and reduced forms of inorganic arsenic led us to suspect that ArsH has a different physiological function other than resistance to inorganic arsenic. Here we report that ArsH confers resistance to the activated trivalent form of the organoarsenical herbicide MSMA and the aromatic arsenical PhAs(III) and not to inorganic species. *Pseudomonas putida* has two chromosomally-encoded *arsRBCH* operons and is highly resistant to the trivalent forms of these organoarsenicals (Canovas *et al.*, 2003), while a derivative with both *arsH* genes deleted is sensitive to MAs(III), PhAs(III) or Rox(III). The amino acid sequences of both PpArsH1 and PpArsH2 are similar to the ArsHs from other organisms (Fig. S1). The gene for PpArsH from the *P. putida arsI* operon (accession number AAN67544.1) was cloned and expressed in the arsenic hypersensitive *E. coli* strain AW3110 (*ars*) (Carlin *et al.*, 1995), where it confers high-level resistance to trivalent MAs(III), PhAs(III) and Rox(III). Purified PpArsH oxidizes these trivalent organical arsenicals to pentavalent species in the order PhAs(III)>Rox(III)>MAs(III). This is the first identification of an organoarsenical oxidase and provides a role for ArsH in the arsenic biogeochemical cycle.

Results

ArsH confers resistance to trivalent organoarsenicals in *P. putida* and *S. meliloti*

The effect of organoarsenicals on *E. coli* W3110, *P. putida* KT2440 and *S. meliloti* Rm1021 was examined. All three were resistant to pentavalent MAs(V), PhAs(V) and Rox(V) (Fig. 1 shows the results for *E. coli* and *P. putida*). *E. coli* exhibited sensitivity to as little as 30 μM MAs(III), 2 μM PhAs(III) and 5 μM Rox(III) (Fig. 1, top), while *P. putida* was approximately 10-fold more resistant to those organoarsenicals (Fig. 1, middle). *P. putida* has two *ars* operons, *ars1* and *ars2*, both of which have *arsRBCH* genes (Paez-Espino *et al.*, 2014). *ars1* has three other genes of unknown function. Deletion of both operons (*P. putida* KT2440 (*ars*)) resulted in sensitivity to MAs(III) and PhAs(III), but not to Rox(III), on both solid (Fig. 1, bottom) and liquid medium (Fig. S2). Similar results were obtained with *S. meliloti*, which is relatively resistant to organoarsenicals (Fig. S3, top). The *ars* operon of *S. meliloti* has *arsR*, *arsC*, *arsH* and *aqpS* (Galibert *et al.*, 2001, Yang *et al.*, 2005), and its deletion (strain Rm310) rendered *S. meliloti* sensitive to the three trivalent organoarsenicals (Fig. S3, middle). Cells of *S. meliloti* SmK958, with deletion of only *arsH*, had the same phenotype (Fig. S3, bottom), suggesting that ArsH confers resistance to organoarsenicals. The *P. putida arsH* gene from *ars1* was cloned and expressed in *E. coli* strain AW3110 (*ars*) (Carlin *et al.*, 1995) under control of the *araC* gene. *E. coli* W3110 (Fig. 2, top) and AW3110 (Fig. 2, middle) are equally sensitive to MAs(III), showing that the *E. coli arsRBC* operon does not confer resistance to MAs(III). In the absence of the inducer arabinose, *E.*

E. coli AW3110 expressing *PpArsH* remained sensitive to MAs(III) but became resistant in the presence of inducer on solid (Fig. 2, bottom and Fig. 3, top) and liquid (Fig. S4, top) medium. Expression of *PpArsH* in *E. coli* AW3110 similarly conferred resistance to PhAs(III) (Fig. 3, middle, Fig. S4, bottom) and Rox(III) (Fig. 3, bottom and Fig. S4 bottom). Since the *ars* operon of *E. coli* AW3110 is deleted, *arsH* expression alone is sufficient to confer resistance to the active trivalent forms of the herbicide MSMA and the poultry antimicrobial growth promoter roxarsone.

PpArsH oxidizes MAs(III), PhAs(III) and Rox(III)

To further investigate the role of PpArsH in trivalent organic arsenic detoxification, the 241-residue *P. putida* ArsH from the *ars1* operon (AAN67544.1) was purified as a C-terminally six-histidine tagged construct with a mass of 28.5 KDa. PpArsH is 81% identical to the 233-residue ArsH encoded by the *ars2* operon, and is closely related to putative ArsH orthologs from other organisms (Fig. S1).

Purified PpArsH is yellow in appearance and exhibits a typical flavoprotein absorption spectrum, with absorption maxima at 373 and 455 nm (Fig. S5). However, purified enzyme contained only 0.17 moles of FMN/mole protein and exhibited significant NADPH:FMN oxidoreductase activity only with exogenously added FMN (V_{\max} of 83 μmol of NADPH oxidized/min/mg protein) (Fig. S6). Neither NADH nor FAD could replace NADPH or FMN. Purified *S. meliloti* ArsH is also yellow, but the occupancy is low, and no FMN density is observed in the crystal structure (Ye *et al.*, 2007). Moreover, the structure exhibits no obvious binding site for arsenicals.

The K_m for NADPH was approximately 74 μM , and the concentration of FMN required for half-maximal activation was 7.8 μM (Fig. S7). No activity was detected with NADH or FAD (Fig. S6). PpArsH exhibited NADPH-dependent FMN reductase activity, but reductase activity was not stimulated by inorganic arsenate or arsenite. PpArsH oxidized MAs(III), PhAs(III) and Rox(III) to the pentavalent arsenical species (Fig. 4), but no oxidation of As(III) was observed (Fig. S8). PpArsH oxidized MAs(III) (Fig. 4, top) and PhAs(III) (Fig. 4, middle) in 30–40 min. The apparent affinity for MAs(III) was approximately 5 μM (Fig. S7). Oxidation of Rox(III) was slower, requiring nearly 3 hrs for complete oxidation (Fig. 4, bottom). The oxidation reaction required both NADPH and FMN (Fig. 5). The reaction was faster with MAs(GS)₂ compared with the unconjugated species, which is not unexpected since most trivalent arsenicals would be expected to be in complex with reduced glutathione (GSH) in the cytosol of cells (Fig. S9). These *in vitro* results are consistent with the results of the *in vivo* resistance assays and demonstrate that ArsH functions as a trivalent organoarsenical oxidase.

The FMN binding site

ArsH orthologs exhibit a high degree of sequence similarity (Fig. S1). The FMN binding site in *S. meliloti* ArsH is predicted to contain the sequence G₁₅SLRTVSYS (Ye *et al.*, 2007). In PpArsH, the corresponding sequence is G₄₂STRERSFS. Alanine substitution of conserved amino acid residues Arg45 and Ser48 reduced resistance to PhAs(III) (Fig. 6). Conserved residue Glu108 is also predicted to contribute to binding of the FMN cofactor. An E108A

mutant did not confer resistance to trivalent organoarsenicals (Fig. 6). In contrast to the yellow wild type PpArsH, purified E108A was colorless and did not have the typical FMN absorption maxima at 373 and 455 nm (Fig. S5). Addition of excess FMN restored 80% of wild type activity to the E108A mutant (Fig. 7) indicating that Glu108 contributes to FMN binding.

The relationship of H₂O₂ formation to MAs(III) oxidation

The ability to oxidize rather than reduce its substrate makes ArsH unique. NADPH:FMN oxidoreductase homologs of ArsH are promiscuous in their enzymatic activity, but their physiological roles are not well defined. For example, closely related FerB from *Paracoccus denitrificans* reduces ferric iron and chromate, but no binding site for a putative substrate is observed in its structure, and its physiological function has not been identified (Sedlacek *et al.*, 2014). Perhaps the reason is because ArsH homologs actually oxidize their true substrates, not reduce them. For example, YhdA from *Bacillus subtilis* oxidizes α,β -unsaturated carbonyl compounds, but this is believed to be the result of indirect and nonenzymatic oxidation by the strong oxidant H₂O₂ produced by YhdA (Mueller *et al.*, 2009).

We considered the possibility that MAs(V) might be produced indirectly as the result of oxidation by H₂O₂. To test this possibility, catalase was added to the reaction to catalyze decomposition of H₂O₂ to H₂O and O₂. Catalase eliminated production of H₂O₂ but not MAs(III) oxidation (Fig. S10), and we conclude that ArsH directly oxidizes MAs(III). Importantly, addition of MAs(III), PhAs(III) or Rox(III) reduced H₂O₂ formation (Fig. S11), suggesting competition for electrons. These results are consistent with H₂O₂ formation being a side reaction and not directly responsible for the majority of MAs(III) oxidation.

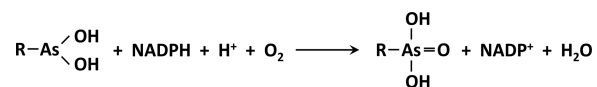
Discussion

The *arsH* gene is found in many arsenic resistance (*ars*) operons, implying a role in arsenic detoxification. Yet cells expressing an *arsH* gene exhibit only low-level resistance to either arsenate or arsenite (Xue *et al.*, 2014, Ye *et al.*, 2007). How, then, does ArsH participate in arsenic resistance? One possibility is that ArsH is a nonspecific reductase that protects cells from oxidative stress (Hervas *et al.*, 2012). If so, why is the *arsH* gene nearly always in *ars* operons? In this study we began with the assumption that *arsH* would not be in an *ars* operon unless it conferred resistance to some form of arsenic. If ArsH does not catalyze a redox reaction with inorganic As(V) or As(III), then a logical conjecture is that the substrate could be an organic form of arsenic. For example, ArsH might confer resistance to the herbicide MAs(V) by reducing it to MAs(III). Yet we found that MAs(V) is not very toxic to bacteria, while MAs(III) is quite toxic, suggesting that the active form of the herbicide is the trivalent species (Chen *et al.*, 2014). However, species with an *arsH* gene in their *ars* operon such as *Pseudomonas putida* KT2440 and *Sinorhizobium meliloti* are resistant to MAs(III), and deletion of the *arsH* gene in these microbes rendered them sensitive. Heterologous expression of an *arsH* gene in *E. coli* conferred resistance to MAs(III). In addition, *arsH* conferred resistance to the reduced forms of aromatic arsenicals such as phenylarsenite and reduced roxarsonate, the poultry antimicrobial growth promoter. Interestingly, cells of *P.*

putida KT2440 with deletion of both *ars* operons remained resistant to Rox(III). One possibility is that *P. putida* lacks an uptake system for Rox(III). Like all *ars* genes, *arsH* is ancient, evolving long before man and the anthropogenic use of herbicides, quite likely arose in response to the production of methylarsenicals by *arsM*-containing bacteria. Just as human use of antibiotics has caused the spread of antibiotic resistance genes, the recent use of methylarsenicals in agriculture may have triggered the spread of organoarsenical resistance genes.

ArsH is a member of the NADPH-dependent FMN oxidoreductase superfamily (Huijbers *et al.*, 2014). It has previously been characterized as a reductase with various substrates, including reduction of azo dyes and O₂ to H₂O₂ (Ye *et al.*, 2007), reduction of Cr(V) to Cr(III) and Fe(III) to Fe(II) (Xue *et al.*, 2014), and one- and two-electron reduction of quinones but not reduction of inorganic As(V) (Hervas *et al.*, 2012). Since the *arsH* gene confers resistance to the trivalent forms of the organoarsenicals, it was more reasonable to consider that the purified enzyme would oxidize them to the relatively less toxic pentavalent species.

No oxidation was observed when NADP⁺ was supplied as an oxidant. In contrast, ArsH oxidized MAs(III), PhAs(III) and Rox(III) when the reductant NADPH was supplied. Mixed-function oxidases use the two atoms of oxygen in O₂ differently, coupling FMN reduction with substrate oxidation (Huijbers *et al.*, 2014). We hypothesize that, rather than being a strict reductase, ArsH catalyzes mixed oxidation-reduction, with reduction of O₂ to H₂O coupled to oxidation of trivalent organoarsenicals. We propose the following reaction scheme for ArsH-catalyzed oxidation of trivalent organoarsenicals, where **R** can be a methyl group (MAs(III)), phenyl group (PhAs(III)) or 3-nitro-4-hydroxyphenyl group (Rox(III)):



In this scheme, the reductive half-reaction consists of formation of an oxidized FMN-NADPH complex followed by a hydride transfer from NADPH to FMN, resulting in FMNH₂ and release of NADP⁺. In the oxidative half-reaction part of the catalytic cycle, an organoarsenical molecule is positioned in proximity of the FMNH₂. The reduced flavin is oxidized by O₂, producing H₂O, followed by oxidation of trivalent arsenical to its pentavalent form. In other words, two electrons from the flavin are transferred to one oxygen atom of dioxygen, reducing it to water, and two electrons from MAs(OH)₂ (MAs(III)) are transferred to the other oxygen atom, forming MAsO(OH)₂ (MAs(V)). ArsH may follow this pathway only when a trivalent organoarsenical substrate is present. In the absence of an organoarsenical substrate, ArsH nonspecifically reduces metals, quinones and azo dyes and can also produce H₂O₂. We consider these reactions to be *in vitro* artifacts that take place only in the absence of physiological substrate. These observations can be explained from the redox potential of the reactions. The mid-point redox potential of the FMN/FMNH₂ couple is -0.27 V. In contrast, the mid-point redox potential of Fe³⁺/Fe²⁺ is +0.77 V, ubiquinone (ox/red) is 0.10 V, and O₂/H₂O is +0.82 V. In the absence of a physiological substrate, the reductive half-reaction of the ArsH catalytic cycle reduces FMN

to FMNH₂ ($E'_{0} = -0.27$ V), while NADPH is oxidized to NADP⁺ ($E'_{0} = +0.32$ V). Electrons then flow from FMNH₂ to nonspecifically reduce the oxidized forms of dyes, metals or quinines, or generate H₂O₂.

Even though ArsH has the ability to reduce azo dyes and chromate, the fact that the ArsH gene is found only in *ars* operons points to an arsenic-related physiological function, and the only high-level activity observed *in vivo* is MAs(III) resistance. The combination of *in vivo* and *in vitro* results clearly demonstrate that the physiological function of ArsH is to confer resistance to trivalent organoarsenicals by oxidizing them to pentavalent species. Various environmental bacterial species in microbial communities reduce MSMA (Yoshinaga *et al.*, 2011), which most likely then become the active herbicide or, at the least, is toxic to other species in the communities (Chen *et al.*, 2014). Bacterial species that carry an *arsH* gene would consequently be resistant to the toxic organoarsenicals produced by their neighbors. The *arsH* lineage predates human invention of herbicides, so the gene must have arisen in response to other environmental pressures. Many members of microbial communities methylate inorganic arsenic to mono- and dimethylated species, which accumulate in the environment as the oxidized species (Ye *et al.*, 2012). We hypothesize that microbial arsenic methylation was the driving force for ArsH evolution.

Materials and Methods

Chemicals

The herbicide MSMA Target 6.6[®] (sodium methanearsonate) was a generous gift from Luxembourg-Pamol (Houston, TX). Roxarsone (3-nitro-4-hydroxyphenylarsonic acid) was purchased from Acros Organics (Fair Lawn, NJ). Unless otherwise indicated, all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO). Pentavalent arsenicals were reduced as described (Reay & Asher, 1977).

Strain, plasmids, medium, and reagents

Strains and plasmids used in this study are described in Table S1. *E. coli* cultures were grown aerobically in Luria-Bertani (LB) or M9 medium at 37 °C supplemented with the appropriate antibiotic (Sambrook *et al.*, 1989). *P. putida* cells were grown aerobically at 30 °C in LB medium, without any antibiotics. For resistance assays, 0.1 ml of fresh cultures of *E. coli* strains (1×10^8 cells) were spread over the surface of M9 minimal agar plates supplemented with 0.5% glycerol (v/v) \pm 0.2% arabinose (w/v) as inducer. Similarly, fresh cultures of *P. putida* strains (1×10^8 cells) were spread onto M9 minimal agar plates supplemented with 0.2% glucose (w/v). After the liquid dried, sterile filter paper disks were applied on to the plates. 10 μ l of each organoarsenical was dispensed on the disks, and the plates were incubated overnight at either 30 °C or 37 °C. The growth rate of cultures of *P. putida* or *E. coli* was monitored from the A_{600nm}. Cultures were grown in LB medium to A_{600nm} of 0.6–0.8, harvested by centrifugation, suspended in M9 minimal medium and inoculated into fresh M9 medium to a final A_{600nm} of 0.004–0.006. The growth of the cultures was monitored over 24 h with organoarsenicals at the indicated concentrations.

Cloning of *P. putida* ArsH

The full-length *P. putida* KT2440 *arsH* (PpArsH; Genbank accession NP_744859) was cloned into the pBAD/*myc*-HisA vector (Life Technologies, Grand Island, NY) so that expression of *arsH* is controlled by the arabinose promoter. The *arsH* gene was amplified by PCR from a *P. putida* genomic DNA library (a gift from Víctor de Lorenzo, Madrid, Spain) to introduce an *Nco*I site at the 5'-end and an *Xho*I site at the 3'-end. The forward and reverse primers were 5'-CATGCCATGGAGACAGAAATGAACGAC-3' and 5'-CCGCTCGAGAATAGACCGAATATTGACTC-3', respectively. The amplified product was gel purified, digested with *Nco*I and *Xho*I, and inserted into the *Nco*I-*Sal*I sites of pBAD/*myc*-HisA vector, in frame with the C-terminal six-histidine-residue tag.

Mutagenesis of the *arsH* gene

Mutations in *P. putida* *arsH* were introduced by site-directed mutagenesis using QuikChange II Site-Directed Mutagenesis Kit from Agilent Technologies (Santa Clara, CA). The mutagenic oligonucleotides used for both strands and the respective changes introduced (underlined) are as follows: R45A, 5'-CTGTACGGATCGACCGCCGAAACGCTCATTCAG-3' and 5'-CTGAATGAGCGTTCGGCGGTCGATCCGTACAG-3'; S48A, 5'-GACCCGCGAACGCGCATTCAGCCGCTT-3' and 5'-AAGCGGCTGAATGCCGGTTTCGCGGTC-3'; E108A, 5'-GTGCTCCCCCGCCGTCACGGCT-3' and 5'-AGCCGTGACGCGCGGGGAGCAC-3'. Each mutation was confirmed by DNA sequencing using Sequetech DNA Sequencing Services (Mountain View, CA).

ArsH purification

E. coli TOP10 cells (Life Technologies) bearing PpArsH in vector plasmid pBAD/*myc*-HisA were grown in Luria-Bertani medium containing 100 µg/ml ampicillin with shaking at 37 °C. At an $A_{600\text{ nm}}$ of 0.6, L-arabinose was added as an inducer to a final concentration of 0.2% (w/v), and the culture was grown for an additional 4 h at 37 °C. The cells were harvested and suspended in 5 ml per gram of wet cells in buffer A (50 mM 4-morpholinepropanesulfonic acid, 20 mM imidazole, 0.5 M NaCl, 10 mM 2-mercaptoethanol and 20% glycerol (vol/vol), pH 7.5). The cells were broken by a single passage through a French pressure cell at 20,000 psi, and immediately treated with diisopropyl fluorophosphate (2.5 µl per gram wet cell). Membranes and unbroken cells were removed by centrifugation at $150,000 \times g$ for 1 h, and the supernatant solution was loaded onto a Ni²⁺-nitrilotriacetic acid column (Qiagen, Valencia, CA) at a flow rate of 0.5 ml/min. The column was washed with more than 25 column volumes of buffer A. PpArsH was eluted with buffer A containing 0.2 M imidazole, and the purity was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). Protein concentrations were estimated from $A_{280\text{ nm}}$ ($\epsilon = 28,120\text{ M}^{-1}\text{ cm}^{-1}$). PpArsH-containing fractions were divided into small portions, rapidly frozen, and stored at -80 °C until use.

Identification of the prosthetic group and assay of FMN reductase activity

The absorption spectrum of both wild type PpArsH and the E108A derivative were compared with a standard solution of 25 μM FMN. PpArsH was assayed for FMN reductase activity in reaction mixtures (1 ml) consisting of 25 mM Bis-Tris Propane (pH 7.0), 0.2 mM NADPH, 25 μM FMN with or without 20 nM ArsH at 37 °C. NADPH oxidation was monitored by the decrease in absorbance at 340 nm ($\epsilon = 6,220 \text{ M}^{-1} \text{ cm}^{-1}$). An Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen) was used to determine H_2O_2 formation (Zhou *et al.*, 1997), and catalase was purchased from Sigma-Aldrich.

Assay of PpArsH activity

P. putida ArsH was assayed for organoarsenical oxidation activity in 1 ml of 25 mM Tris, 25 mM BIS-Tris Propane (pH 7.0), 1 mM EDTA, 0.2 mM NADPH, 25 μM FMN, 4 μM organoarsenicals and 0.1 μM PpArsH at 37 °C. At the indicated times, a 0.1 ml portion was filtered through 3-kDa cutoff Amicon Ultrafilter (EMD Millipore, Billerica, MA). The filtrate was then speciated by high performance liquid chromatography (HPLC) (Series 2000; Perkin-Elmer, Waltham, MA) coupled to inductively coupled plasma mass spectroscopy (ICP-MS) (ELAN DRC-e; Perkin-Elmer) using either a Jupiter® 5 μm C18 300 Å reverse-phase column (250 mm \times 4.6 mm; Phenomenex, Torrance, CA) eluted isocratically with a mobile phase consisting of 3 mM malonic acid, 5 mM tetrabutylammonium hydroxide, and 5% methanol (v/v), pH 5.6, with a flow rate of 1 ml/min at 25 °C or an Inertsil® 5 μm C4 150 Å reverse-phase column (150 mm \times 2.1 mm; GL Sciences, Torrance, CA) eluted with 15% acetonitrile (v/v), 15% ethanol (v/v), 80% water (v/v), pH 1.5, with a flow rate of 0.8 ml/min at 60 °C.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

MAs(III)	Methylarsenite
MAs(GS)₂	methylarsenite diglutathione
MAs(V) or MSMA	methylarsenate
DMA(V)	dimethylarsenate
PhAs(V)	phenylarsenate
PhAs(III)	phenylarsenite
Rox(V)	roxarsone 3-nitro-4-hydroxybenzenearsonic acid or
Rox(III)	roxarsone with reduced As(III)

(HPLC)	high pressure liquid chromatography
(ICP-MS)	inductively coupled plasma mass spectroscopy
FMN	flavin mononucleotide
GSH	reduced glutathione.

References

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Summary

The *arsH* gene found in many arsenic resistance (*ars*) operons encodes an atypical flavodoxin, but the physiological role of ArsH in arsenic resistance has been unclear. Here we demonstrate that ArsH is an organoarsenical oxidase that detoxifies the trivalent forms of the herbicide sodium methylarsenate (MSMA) and the poultry antimicrobial growth promoter roxarsone by oxidation to pentavalent species. Our results suggest that ArsH catalyzes a novel biotransformation that confers resistance to environmental methylated and aromatic arsenicals.

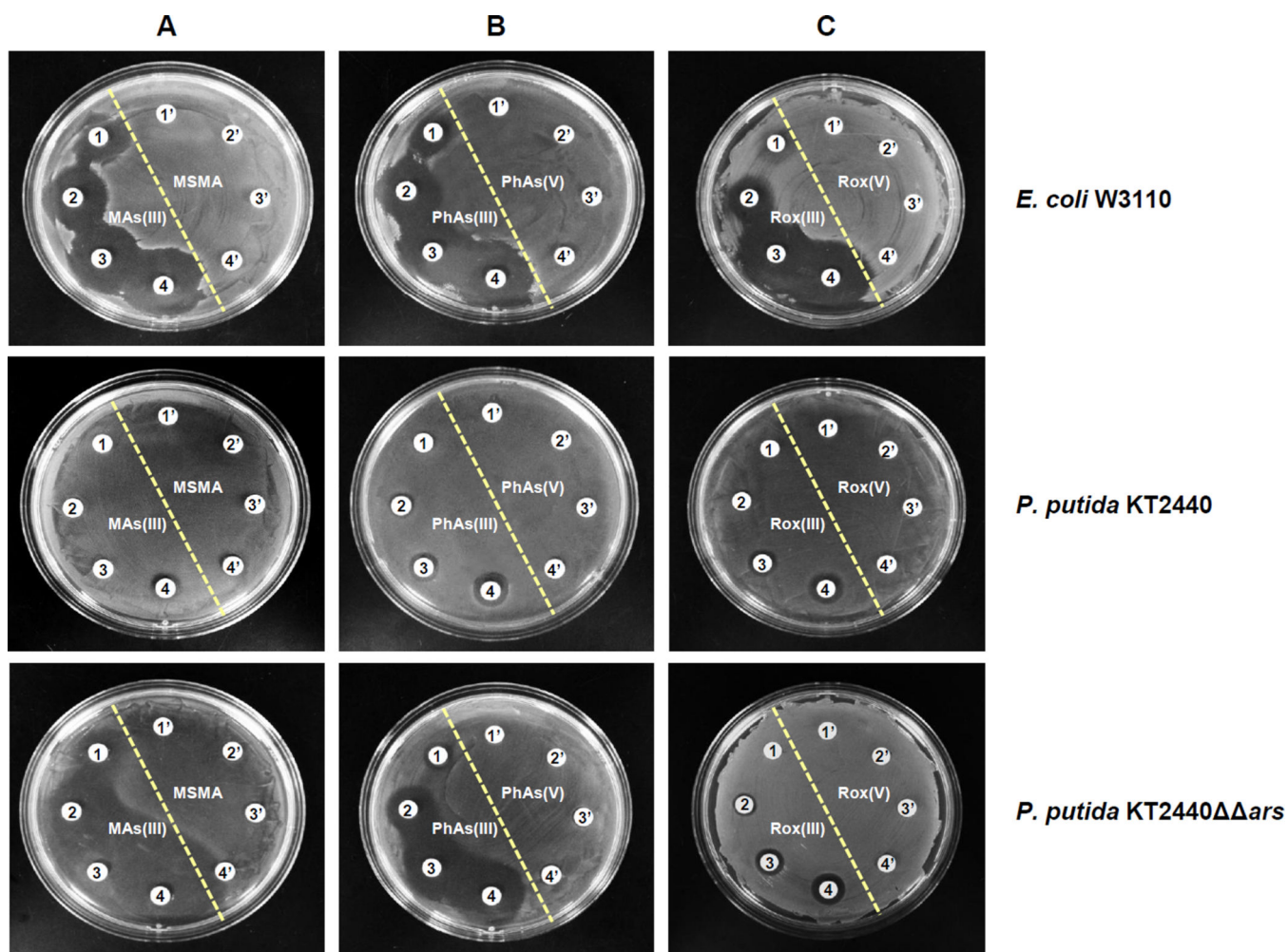


Fig. 1. Resistance to MAS(III), PhAs(III) or Rox(III) of *E. coli* (top), *P. putida* wild type (middle) and *P. putida ars* (bottom)

The effects of pentavalent and trivalent organoarsenicals on cells grown on M9 agar plates overnight were compared. A: 1') no addition; 2') 40 μ M MSMA; 3') 50 μ M MSMA; 4') 60 μ M MSMA; 1) 30 μ M MAS(III); 2) 40 μ M MAS(III); 3) 50 μ M MAS(III); 4) 60 μ M MAS(III). B: 1') no addition; 2') 5 μ M PhAs(V); 3') 10 μ M PhAs(V); 4') 15 μ M PhAs(V); 1) 2 μ M PhAs(III); 2) 5 μ M PhAs(III); 3) 10 μ M PhAs(III); 4) 15 μ M PhAs(III). C: 1') no addition; 2') 10 μ M Rox(V); 3') 15 μ M Rox(V); 4') 20 μ M Rox(V); 1) 5 μ M Rox(III); 1) 10 μ M Rox(III); 3) 15 μ M Rox(III); 4) 20 μ M Rox(III).

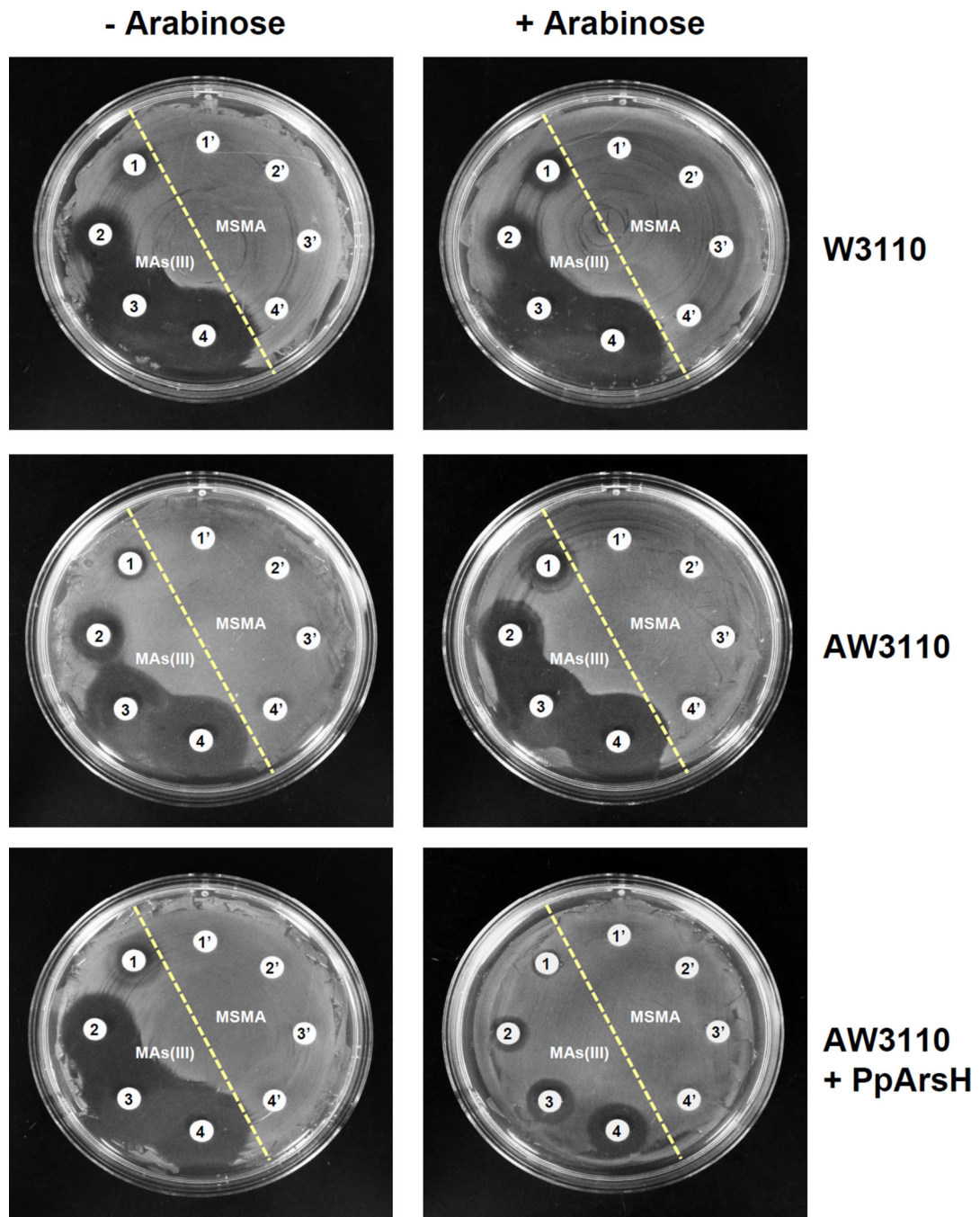


Fig. 2. PpArSH confers resistance to MAs(III) in *E. coli*

The effects of MAs(III) on the growth of *E. coli* W3110 (wild type) (top), AW3110 *ars* (middle) or AW3110 expressing the *PpArSH* gene (bottom) were grown on M9 agar plates overnight with the indicated concentrations of methyl arsenicals at 37 °C. *PpArSH* was under control of the *ara* promoter in vector plasmid pBAD, which was uninduced or induced by 0.2% arabinose. 1') no addition; 2') 40 μM MSMA; 3') 50 μM MSMA; 4') 60 μM MSMA; 1) 30 μM MAs(III); 2) 40 μM MAs(III); 3) 50 μM MAs(III); 4) 60 μM MAs(III).

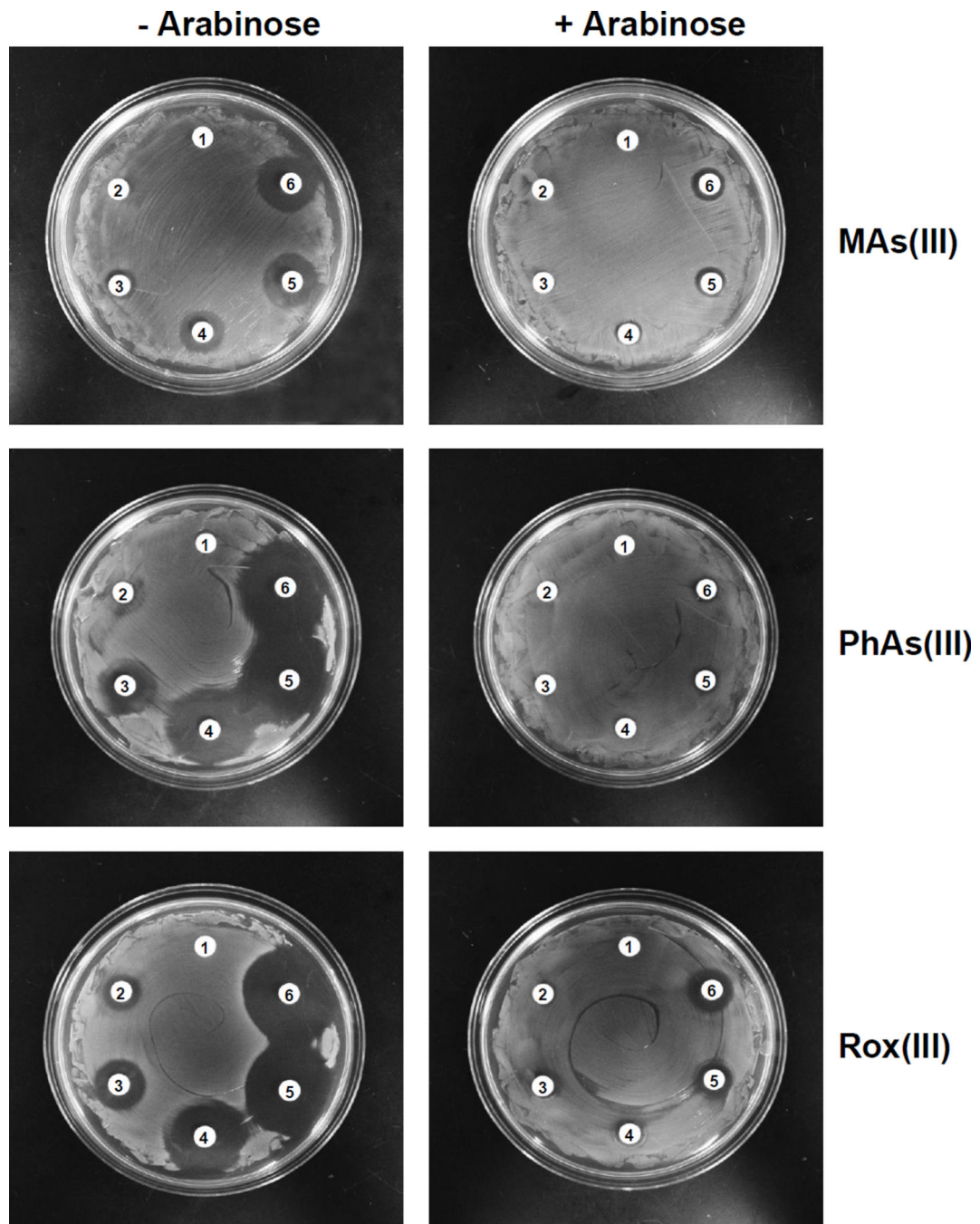


Fig. 3. Heterologous expression of *PparsH* confers resistance to trivalent organoarsenicals
 Cells of *E. coli* AW3110 expressing the *PparsH* gene were grown on M9 agar plates with the indicated concentrations of organoarsenicals at 37 °C. The *PparsH* gene was either uninduced (left) or induced with 0.2% arabinose (right). Top: MAs(III) concentration 1) none, 2) 5 μ M, 3) 15 μ M, 4) 25 μ M, 5) 35 μ M, 6) 45 μ M. Middle: PhAs(III) 1) none, 2) 2 μ M; 3) 5 μ M, 4) 10 μ M, 5) 15 μ M, 6) 20 μ M. Bottom: Rox(III) concentration 1) none, 2) 5 μ M, 3) 10 μ M, 4) 15 μ M, 5) 20 μ M, 6) 25 μ M.

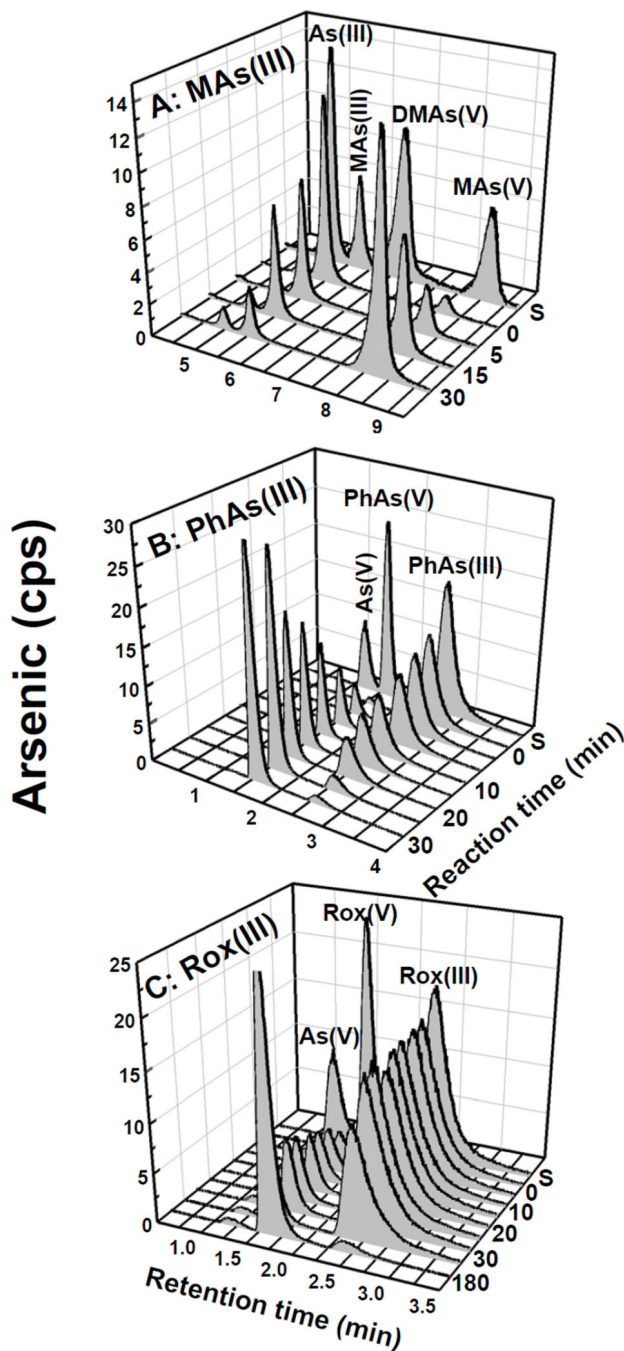


Fig. 4. Time course of oxidation of trivalent organoarsenicals by PpArsH

Oxidation of organoarsenicals by purified PpArsH was assayed as described in Material and Methods. **A:** 4 μM MAs(III) and 0.5 μM PpArsH; **B:** 4 μM PhAs(III) and 0.1 μM ArsH; **C:** 4 μM Rox(III) and 0.1 μM ArsH. Samples were separated by HPLC using either a C18 (MAs(III)) or C4 (PhAs(III) and Rox(III)) reverse phase column, and the amount of arsenic in relative counts per second (cps) was estimated by ICP-MS.

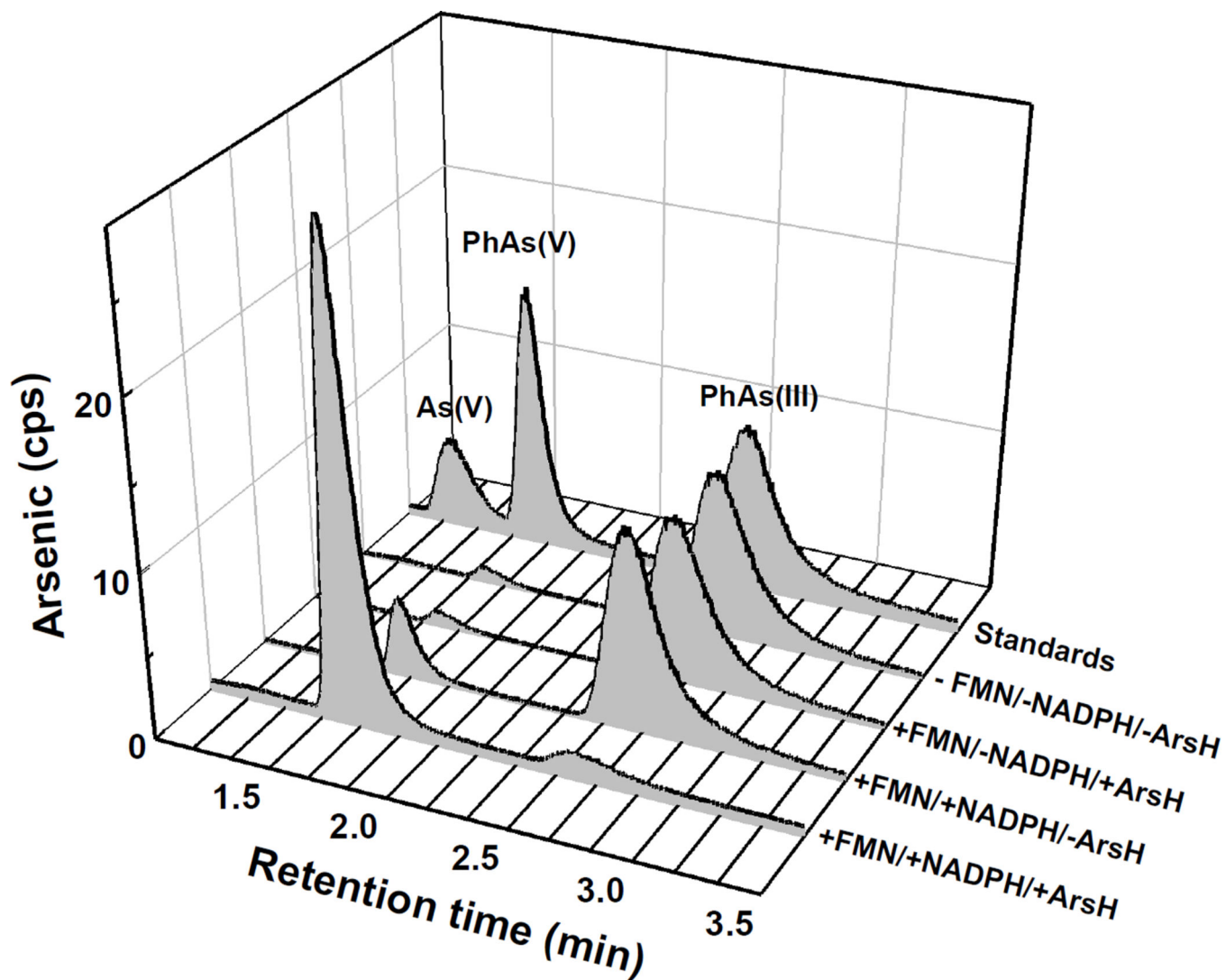


Fig. 5. NADPH and FMN are required for ArsH catalysis

PhAs(III) oxidation by PpArsH was assayed with or without NADPH and/or FMN, as described in Materials and Methods. The reaction mixture contained 0.1 μM ArsH, 4 μM PhAs(III), 0.2 mM NADPH and 25 μM FMN. Samples were separated by HPLC using a C4 reverse phase column, and the amount of arsenic in relative counts per second (cps) was estimated by ICP-MS.

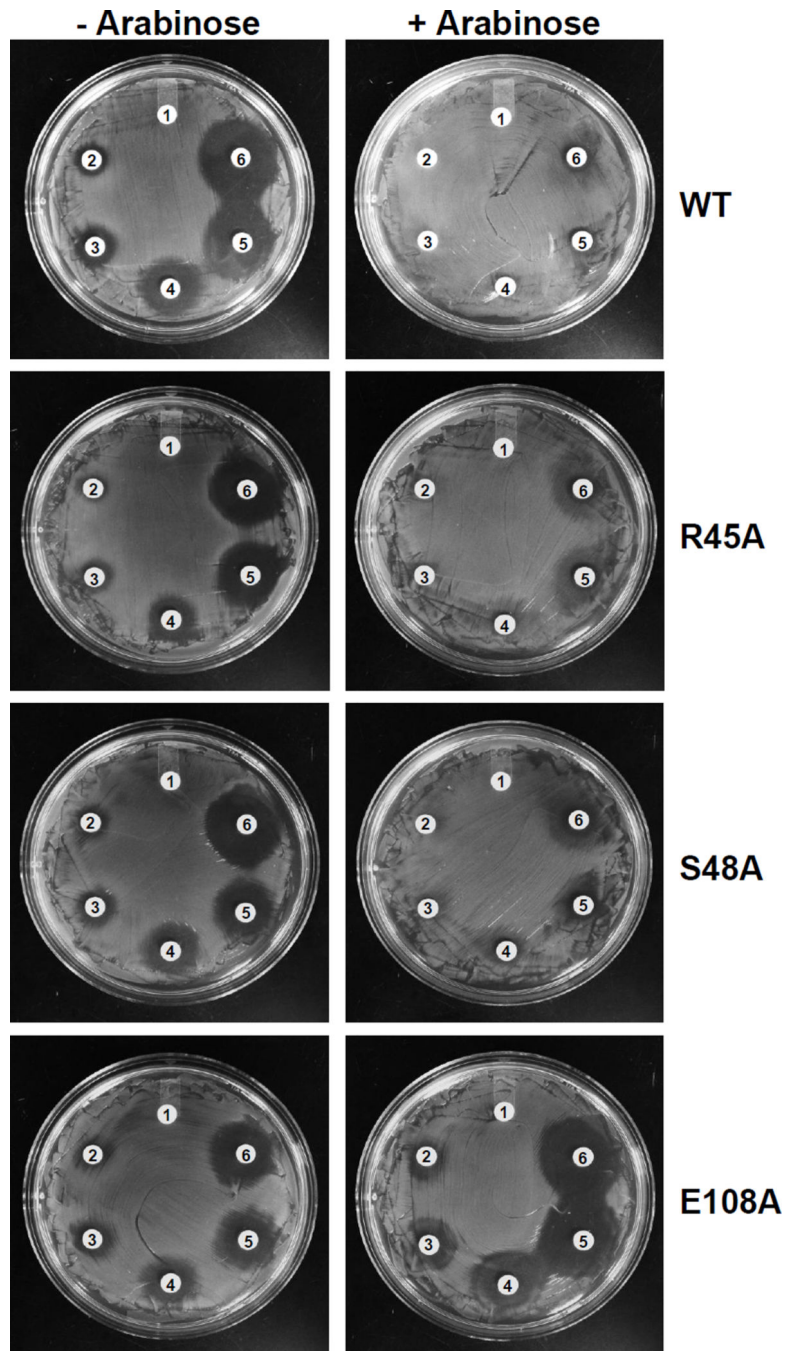


Fig. 6. PhAs(III) resistance of PpArsH mutants

Cells of *E. coli* AW3110 expressing the wild type *PpArsH* (top) or mutants R45A (2nd row), S48A (3rd row) or E108A (bottom) were grown on M9 agar plates with the indicated concentrations PhAs(III) overnight at 37 °C. Cells were uninduced (left) or induced with by 0.2% arabinose (right). PhAs(III) concentration: 1) none, 2) 2 μM, 3) 4 μM, 4) 6 μM, 5) 8 μM, 6) 10 μM.

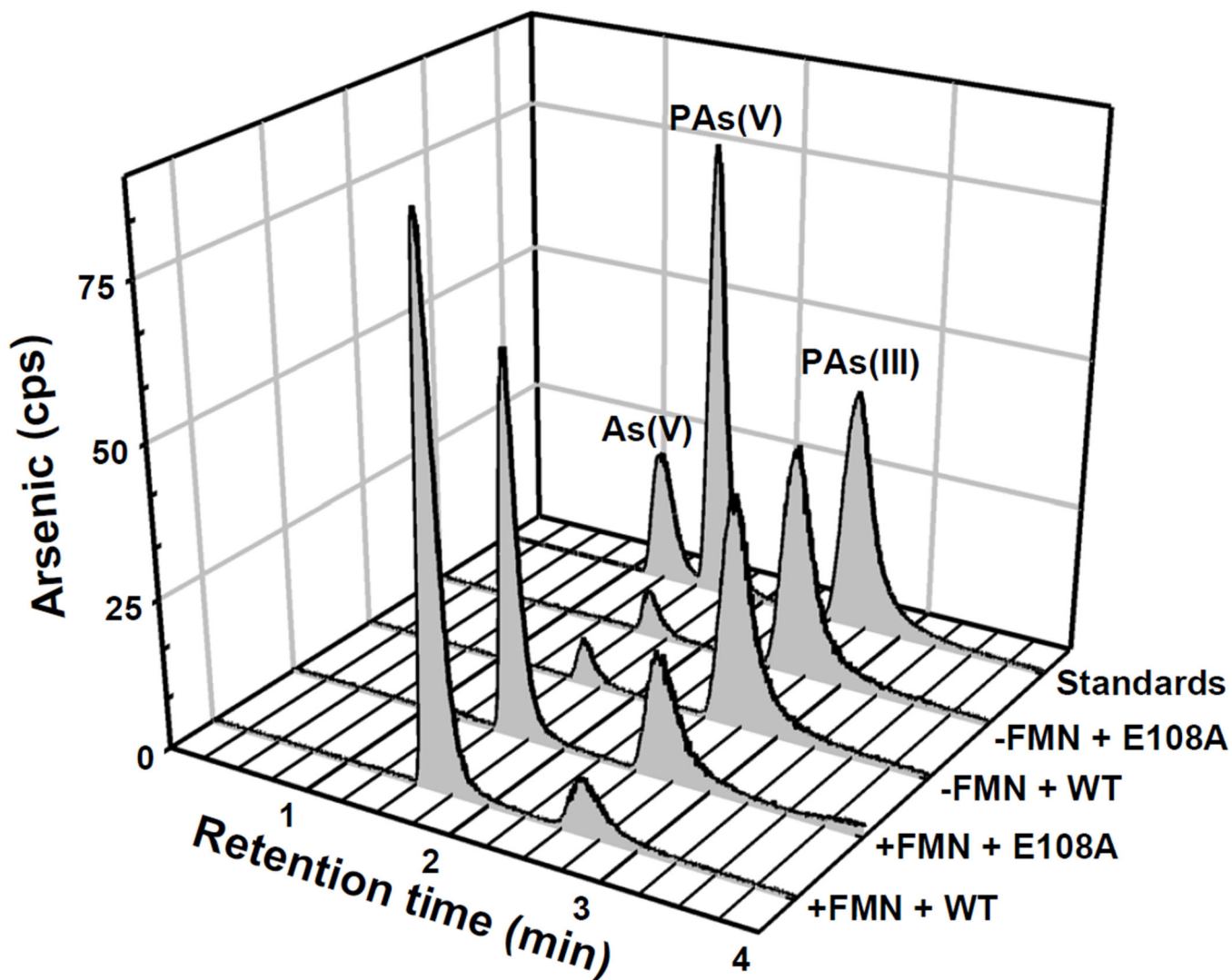


Fig. 7. FMN restores PhAs(III) oxidation in an E108A mutant

0.1 μ M E108A ArsH was incubated with 4 μ M PhAs(III), 0.2 mM NADPH and 75 μ M FMN, and the reaction was monitored as described in Materials and Methods. Samples were separated by HPLC using a C4 reverse phase column, and the amount of arsenic in relative counts per second (cps) was estimated by ICP-MS.