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Organoarsenical Biotransformations by *Shewanella putrefaciens*

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Abstract

Microbes play a critical role in the global arsenic biogeochemical cycle. Most studies have focused on redox cycling of inorganic arsenic in bacteria and archaea. The parallel cycles of organoarsenical biotransformations are less well characterized. Here we describe organoarsenical biotransformations in the environmental microbe *Shewanella putrefaciens*. Under aerobic growth conditions, *S. putrefaciens* reduced the herbicide MSMA (methylarsenate or MAs(V)) to methylarsenite (MAs(III)). Even though it does not contain an *arsI* gene, which encodes the ArsI C–As lyase, *S. putrefaciens* demethylated MAs(III) to As(III). It cleaved the C–As bond in aromatic arsenicals such as the trivalent forms of the antimicrobial agents roxarsone (Rox(III)), nitarsones (Nit(III)) and phenylarsenite (PhAs-(III)), which have been used as growth promoters for poultry and swine. *S. putrefaciens* thiolated methylated arsenicals, converting MAs(V) into the more toxic metabolite monomethyl monothioarsenate (MMMTAs(V)), and transformed dimethylarsenate (DMAs(V)) into dimethylmonothioarsenate (DMMTAs(V)). It also reduced the nitro groups of Nit(V), forming *p*-aminophenyl arsenate (*p*-arsanilic acid or *p*-AsA(V)), and Rox(III), forming 3-amino-4-hydroxybenzylarsenate (3A4HBzAs(V)). Elucidation of organoarsenical biotransformations by *S. putrefaciens* provides a holistic appreciation of how these environmental pollutants are degraded.

Graphical Abstract

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ASSOCIATED CONTENT

Supporting Information

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INTRODUCTION

The presence of arsenic resistance (*ars*) genes in the genome of nearly every living organism sequenced to date suggests that most organisms are continually exposed to this ubiquitous environmental toxin. The majority of characterized *ars* genes encode proteins involved in sensing, reduction or transport of inorganic arsenic.¹ Recently the existence of a parallel cycle for biotransformations and detoxification of highly toxic methylated and aromatic organoarsenicals has been recognized.¹ In the environment methylarsenite (MAs(III)) is both synthesized and broken down, and there is a redox cycle between relatively nontoxic oxidized methylarsenate (MAs(V)) and highly toxic reduced MAs(III).² A few bacterial species use organoarsenicals as source of carbon.³ In addition, under anoxic conditions, some bacteria, including anaerobic sulfate-reducing gut microbe bacteria, produce a variety of highly toxic methylated thioarsenicals including monomethyl monothioarsenate (MMMTAs(V)) and dimethylmonothioarsenate (DMMTAs(V)).^{4,5} Thioarsenicals, which are very toxic, are found in human and animal urine, but it is unclear whether these thioarsenicals are produced by animals or by their microbiome. Nor is the mechanism of microbial thioarsenical formation known, and it is not clear if this is an enzymatic process.^{5–8}

Organoarsenicals are synthesized biologically and are introduced into the environment anthropogenically.¹ Many organoarsenicals are degraded by microbes into inorganic arsenic, which contaminates our food and water supplies. The herbicide MSMA (MAs(V)) is degraded by microbial communities into As(III), which is more toxic and carcinogenic than MSMA.⁹ Roxarsone, an organoarsenical antimicrobial growth promoter for poultry and swine, is microbially degraded to 4-hydroxy-3-aminophenylarsonic acid^{10,11} and eventually to inorganic arsenic.¹²

Recently microbial *ars* operons encoding enzymes that catalyze a variety of organoarsenical biotransformations have been identified and cloned. The *arsI* gene was cloned from a soil bacillus.¹² The gene product, ArsI, is a C–As bond lyase, a member of the dioxygenase superfamily, that detoxifies MAs(III) by cleaving it into As(III) and H₂CO. The *arsH* gene was cloned from a rhizosphere bacterium, and its gene product, ArsH, is an NADPH-FMN

dependent oxidoreductase that oxidizes highly toxic MAs(III) to relatively nontoxic MAs(V).¹³ The *arsP* gene was cloned from *Campylobacter jejuni*, which is found in animal feces and is one of the major agents of food poisoning. ArsP is an efflux pump for MAs(III) and roxarsone that allows *C. jejuni* to survive in roxarsone-treated poultry and swine.¹⁴ All of these genes of arsenic biotransformation are found in arsenic resistance (*ars*) operons, the most widespread resistance determinants in nature. In total, all of these microbial pathways are fundamental components of the global arsenic biogeochemical cycle.

The objective of this study was to understand how microbial biotransformations contribute to degradation of environmental pollutants such as organoarsenical herbicides and antimicrobial growth promoters. Well-characterized *ars* operons include genes that encode resistance to inorganic As(V) and As(III), as well as respiratory reductases and oxidases that generate energy from inorganic arsenicals.¹ These genes are found in many bacteria, including *Shewanella* species, a group of metabolically adaptable facultative anaerobic bacteria microorganisms found in seawater, freshwater, soil and food.^{15–17} In this study we used *Shewanella putrefaciens* as a model because it is a highly versatile environmental microbe that catalyzes rapid dissimilatory reduction of ferric iron, and Fe(III)-reducing *Shewanella* sp. are found in agricultural soils at concentrations up to 10⁵ cells per gram. *Shewanella* species are capable of a variety of organoarsenical biotransformations with a broad range of substrates. It uses As(V) as a terminal electron acceptor for anaerobic respiration.^{18,19} To date there little is known about the ability of *Shewanella* to transform or detoxify organoarsenicals and thioarsenicals.

In this study, we examined the biotransformation profile of organoarsenicals by *S. putrefaciens* 200, which was originally isolated from a Canadian oil pipeline but inhabits a wide range of marine and terrestrial environments and can be a food contaminant.^{18,20,21} *Shewanella* can utilize a wide variety of metals as electron acceptors during anaerobic respiration, which has made it useful for bioremediation of metal contaminants.²² During anaerobic growth *S. putrefaciens* can reduce As(V) to As(III).²³ Here we demonstrate that *S. putrefaciens* 200 has pathways for biotransformations of a broad range of environmental organoarsenicals, including the herbicide MSMA (MAs(V)) and the avian and porcine antimicrobial growth promoters roxarsone, nitarosone and arsanilic acid. Additionally *S. putrefaciens* catalyzed aerobic thiolation of MAs(V) and dimethylarsenate (DMAs(V)), transforming them into MMTAs(V) and DMMTAs(V), respectively. This is the first identification of an isolated single environmental microbe capable of thiolating methylated arsenicals, and the ability to do so in air is unprecedented. These results provide new insights into the contribution of this widespread environmental facultative anaerobe to the arsenic biogeochemical cycle.

MATERIALS AND METHODS

Chemicals

Unless otherwise indicated, chemicals were purchased from Sigma-Aldrich. Roxarsone (Rox(V)) and MAs(V) were obtained from ThermoFisher Acros Organics Division (Waltham, MA) and Chem Service (West Chester, PA) respectively. Phenylarsenite (PhAs(III) or PAO), nitarosone (Nit(V)), *p*-arsanilic acid (*p*AsA(V)) and 3-amino-4-

hydroxybenzylarsonate (3A4HBzAs(V)) were purchased from Sigma-Aldrich (St Louis, MO). Pentavalent arsenicals were reduced as described.²⁴ The reduced products were not thiolated, as determined by simultaneous As and S analysis by high pressure liquid chromatography (HPLC) coupled with inductively coupled mass spectroscopy (ICP-MS) (ELAN DRC-e; PerkinElmer, Waltham, MA).²⁵ The structure of the arsenical compounds described in this study are shown in Supporting Information Table 1S.

Strains, medium and growth conditions

S. putrefaciens 200 used in this study was a gift from Flynn Picardal, Indiana University. Unless otherwise noted, cultures of *S. putrefaciens* were grown aerobically in Luria–Bertani (LB) medium, M9 medium²⁶ or ST 10⁻¹ medium (0.5 g/L peptone and 50 mg/L yeast extract)²⁷ at 30 °C with shaking. Bacterial growth was monitored by measuring the absorbance at 600 nm ($A_{600\text{ nm}}$).

Organoarsenicals Biotransformation by *S. putrefaciens* 200

To analyze methylarsenicals biotransformation, *S. putrefaciens* was cultured aerobically with shaking in LB medium overnight at 30 °C. After washing the cells once with ST 10⁻¹ medium supplemented with 0.2% D-glucose, cells was suspended in M9 medium or ST 10⁻¹ medium and cell density was adjusted to $A_{600} = 3.0$. Organoarsenicals were individually added at 4 μM , final concentration, to the cell suspensions, which were incubated at 30 °C with shaking for 4 h. Soluble organoarsenicals were speciated by high-performance liquid chromatography (HPLC) (Series 2000; PerkinElmer, Waltham, MA) coupled to inductively coupled plasma mass spectroscopy (ICP-MS) (ELAN DRC-e; PerkinElmer) 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⁻¹ 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⁻¹ at 60 °C. The choice of reverse phase column depended on chemical species to be separated, and the elution time and profile dependent on the column. Nearly all of the arsenic was recovered in these experiments.

Preparation of Thioarsenicals

A MMTAs(V) standard was prepared as described.²⁸ A 126 μM MAs(V) solution was prepared by mixing 60 μL of 6.3 mM MAs(V) with 2.94 mL of 10% (v/v) formic acid. A saturated H₂S solution was prepared by adding 2 mL of HCl in 4 mL of double-deionized water to 1.0 g of FeS. The released H₂S gas was captured into 15 mL of double-deionized water until the effervescence in the round-bottom flask subsided. The saturated H₂S solution (0.1 mL) was added to 0.9 mL of the 126 μM MAs(V) solution in a 1 mL glass vial and shaken overnight. The progress of the reaction was verified by HPLC-ICP-MS. A DMMTAs(V) standard was prepared as described.²⁹ Briefly, DMMTAs(V) was prepared by stepwise addition of concentrated H₂SO₄ to an aqueous solution of 38 mM DMAs(V) and 60 mM Na₂S at a final molar ratio of DMAs(V):Na₂S:H₂SO₄ = 1:1.6:1.6. The solution was allowed to stand for 1 h, and DMMTAs(V) was extracted with diethyl ether.

Organoarsenical Uptake by *S. putrefaciens* 200

For in vivo uptake assays, cultures of *S. putrefaciens* were grown to an $A_{600\text{ nm}} = 2$ at 30 °C with aeration in LB medium. The cells were harvested and suspended in 20% of the original medium volume in a buffer consisting of 75 mM HEPES-KOH, pH 7.5, 0.15 M KCl and 1 mM MgSO_4 . For induction experiments, it was necessary to modify the growth conditions because MAs(III) is unstable in LB medium. MAs(III) is stable for several days in a medium consisting 5-fold concentrated ST 10^{-1} medium supplemented with 0.2% D-glucose, which was used to grow cells induced with MAs(III) or As(III). To initiate transport reactions, organoarsenicals were added at 10 μM , final concentration, to 1 mL of cell suspension. Portions (0.2 mL) were withdrawn after 45 min, filtered through nitrocellulose filters (0.2 μm pore diameter; EMD Millipore, Billerica, MA) and washed twice with 5 mL of the same buffer at room temperature. The filters were digested with 0.3 mL of concentrated HNO_3 (68–70%) overnight at room temperature. The dissolved filters were incubated for 10 min at 70 °C, allowed to cool to room temperature and diluted with HPLC-grade water (Sigma-Aldrich) to produce a final HNO_3 concentration of 2%. Particulate matter was removed by centrifugation, and arsenic was quantified by ICP-MS. Standard solutions were made in the range of 0.5–50 ppb in 2% nitric acid using an arsenic standard (Ultra Scientific, N. Kingstown, RI). Protein content was determined using a Pierce BCA Protein Assay Kit (Life Technologies).

RESULTS AND DISCUSSION

Organoarsenical Resistance Genes in *S. putrefaciens* 200

In the chromosome of *S. putrefaciens* 200 (NCBI accession number AE014299.2) is a cluster of 19 genes, of which only four encode proteins shown to be involved in organoarsenical detoxification (Figure 1S). Two genes encode ArsP efflux permeases that provide resistance to the herbicide MSMA and the poultry growth promoter roxarsone.¹⁴ The product of the *gapdh* gene, glyceraldehydes-3-phosphate dehydrogenase arsenylates glyceraldehydes-3-phosphate to 1-arseno-3-phosphoglycerate, the substrate of the *arsJ* gene product, the ArsJ efflux permease.³⁰ Other known organoarsenical biotransformation genes are absent from the genome of *S. putrefaciens* 200. These include a gene for the ArsM As(III) S-adenosylmethionine methyltransferase,²⁵ and *S. putrefaciens* 200 did not methylate As(III) (data not shown). Also absent is the gene for the ArsH MAs(III) oxidase,¹³ and no MAs(III) oxidation was observed. The genome also lacks the gene for the ArsI MAs(III) demethylase.¹² This is curious because, as described below, *S. putrefaciens* demethylates MAs(III), suggesting a novel ArsI-independent reaction. To identify the pathways of organoarsenical biotransformations that this versatile microbe can carry out, we analyzed the end products of metabolism of a select group of organoarsenicals (Supporting Information Table 1S).

Methylarsenical Biotransformations

The first question was the fate of MAs(III) and MAs(V). Methylarsenical biotransformations by *S. putrefaciens* 200 were examined following growth in minimal M9 medium. While arsenic thiolation is usually associated with anaerobes, when *S. putrefaciens* was incubated aerobically with 4 μM MAs(III), after 4 h, $17.9 \pm 1.30\%$ was converted to the thioarsenical

MMMTAs(V), and $29.6 \pm 1.5\%$ was demethylated to As(III) (Table 1). After 12 h, the amount of MMTAs(V) was constant, but $80.5 \pm 3.3\%$ of the MAs(III) was transformed into As(III). Thus, demethylation was more rapid than thiolation, which limited the amount of MMTAs(V) synthesized. These results demonstrate that *S. putrefaciens* has parallel aerobic pathways of MAs(III) transformation, slow thiolation, and rapid demethylation. Since there is no *arsI* gene in the chromosome, *S. putrefaciens* may have a different pathway for cleavage of the C–As bond. When *S. putrefaciens* was incubated with $4 \mu\text{M}$ MAs(V), $20.8 \pm 1.8\%$ was reduced to MAs(III) after 4 h, and finally, $77.5 \pm 2.3\%$ of MAs(V) was demethylated to As(III) with $5.3 \pm 0.3\%$ MMTAs(V) after 12 h. This indicates that MAs(V) biotransformation is a two-step process of MAs(V) reduction and MAs(III) demethylation, as we have previously shown in soil microbes.⁹ DMAs(III) is unstable, so we could not determine the fate of DMAs(III), but no reduction or demethylation of DMAs(V) was observed during aerobic incubation. $81.0 \pm 3.3\%$ of DMAs(V) was thiolated to DMMTAs(V). It appears that *S. putrefaciens* did not reduce DMAs(V), so demethylation of DMAs(III) could not be determined, and, in the absence of a competing pathway, the pathway of DMAs(V) thiolation predominated.

What is the source of sulfur for arsenic thiolation by *S. putrefaciens*? While formation of thiomethylarsenicals appears to be biological, it is not clear whether it is an enzymatic process or simply a nonenzymatic chemical reaction of methylated arsenicals with biologically produced hydrogen sulfide. *S. putrefaciens* has a chromosomally encoded *phsA* gene for a putative PhsA thiosulfater reductase (accession number WP_014609795.1) that catalyzes anaerobic production of hydrogen sulfide from thiosulfate. Bacterial arsenic thiolation is associated with sulfate reduction under anaerobic conditions.³¹ Thioarsenicals are formed in sulfidic solutions, including hot springs³² and the anaerobic large intestine,⁵ where hydrogen sulfide is produced. Methylarsenic thiolation is formed in anaerobic mixed cultures such as those found in gut microbiomes.^{6,31} Rats fed As(III) excrete thiomethylarsenicals in their urine.³³ Liver homogenates form DMMTAs(V) from DMAs(III) in the presence of thiosulfate.³⁴ Mice with an AS3MT gene disruption do not methylate As(III) and do not excrete MMTAs(V) or DMMTAs(V) in urine, suggesting a link between arsenic methylation and formation of highly toxic thiomethylarsenicals.^{4,35} Since *S. putrefaciens* has a *phsA* gene, the organism should be capable of producing hydrogen sulfide. We predict that H_2S produced by *S. putrefaciens* reacted with the methylated arsenicals to transform MAs(V) into the more toxic metabolite MMTAs(V), and DMAs(V) into DMMTAs(V). This is the first demonstration of aerobic production of thioarsenicals.

Aromatic Arsenicals Biotransformations

The above results demonstrate the ability of *S. putrefaciens* to transform simple methylarsenicals. However, more complex aromatic arsenicals, including roxarsone (Rox(V)), nitarsone (Nit(V)) and phenylarsonic acid (PhAs(V)) have been used previously in the United States and are still used today in countries such as China and India as antimicrobial growth enhancers in animal husbandry, so the ability of organisms such as *S. putrefaciens* to transform or degrade aromatic arsenicals is an important environmental issue in farming regions. Roxarsone passes through the intestinal track of poultry and swine

mostly unmodified but is subsequently degraded to inorganic arsenic after composting and use as fertilizer.^{10,11,36} Anaerobic bacteria have been shown to be involved in degradation.¹¹ To better understand the fate of these environmental contaminants, biotransformation by *S. putrefaciens* was examined (Table 2). When *S. putrefaciens* was incubated with either 4 μ M Rox(III) or Rox(V), Rox(V) was not altered after 12 h, while Rox(III) was converted to a mixture of inorganic As(III), Rox(V) and 3-amino-4-hydroxybenzylarsonate (3A4HBzsAs(V)). Rox(III) is more toxic than As(III),³⁷ so that C–As bond cleavage is a detoxification process. It is not clear whether Rox(III) oxidation was enzymatic, or the result of aerobic incubation. The fate of other trivalent aromatic arsenicals, including PhAs(III) and Nit(III) was also examined (Table 2). $25.5 \pm 1.3\%$ of Nit(III) was converted into inorganic As(III). PhAs(III) was not transformed into As(III), but three unknown peaks were observed. There was no metabolism of PhAs(V) after 12 h of incubation. These results clearly demonstrate that *S. putrefaciens* has the potential to contribute to degradation of complex organoarsenicals such as those present in the soil of farming regions.

Reduction of the Nitro Group of Aromatic Arsenicals

Does *S. putrefaciens* modify aromatic arsenicals in ways other than cleavage of the C–As bond? In microbial degradation of roxarsone, reduction of the nitro group to an amine has been observed.³⁸ To explore the ability of *S. putrefaciens* to reduce the nitro group, cells were incubated with 4 μ M Nit(V) for 12 h. The Nit(V) peak decreased, and a new peak appeared at the elution position of *p*-arsanilic acid (*p*AsA(V)), in which the aromatic nitro substituent was reduced to an amine (Table 2). After 12 h incubation, $14.8 \pm 1.0\%$ of Rox(III) was converted to 3A4HBzsAs(V). $51.0 \pm 3.8\%$ of Nit(III) was reduced to *p*AsAs(V). $78.5 \pm 3.0\%$ of Nit(V) was reduced to *p*AsA(V) and $5.5 \pm 0.8\%$ was converted to inorganic As(III). Thus, *S. putrefaciens* reduced both the pentavalent arsenic atom and the nitro substituent in aromatic arsenicals. Thus, this single organism is capable of multiple modifications of aromatic arsenical growth promoters.

Organoarsenical Biotransformations in Marine Environments Are Different from Those in Low Salt Environments

S. putrefaciens is present in many environments, including soil, fresh water, and seawater. We asked whether it might carry out different arsenic biotransformations in marine environments than in fresh water environments. To approximate these conditions, cells were grown in media with high or low salt. When cultures were incubated in M9 medium, which contains 93 mM sodium salts and 22 mM potassium salts, MAs(III) was demethylated to As(III) and oxidized to MAs(V) (Table 3). MAs(V) was reduced to MAs(III) and demethylated, with some thiolation to MMTAs(V). In addition, DMAs(V) was thiolated to DMMTAs(V). In contrast, when the cells grown in LB medium, washed and incubated in the low salt ST 10^{-1} medium that had been designed for isolation of MAs(III) demethylating bacteria,²⁷ neither MAs(V) nor DMAs(V) were metabolized. On the other hand, demethylation of MAs(III) was comparable in M9 and ST 10^{-1} medium. The requirement for high salt, for additional carbon and/or for osmolarity for methylarsenical biotransformations was examined by supplementing ST 10^{-1} medium with either 0.1 M NaCl or 0.1 M glucose. Cells incubated in ST 10^{-1} medium supplemented with 0.1 M NaCl exhibited reduction of MAs(V) to MAs(III), demethylation to As(III) and thiolation to

MMMTAs(V) compared to medium without NaCl. $79.8 \pm 3.8\%$ of DMAs(V) was thiolated to DMMTAs(V), but only when the medium was supplemented with 0.1 M NaCl. Cells incubated in ST 10^{-1} medium supplemented with 0.1 M glucose did not transform either MAs(V) or DMAs(V), indicating that neither higher osmolarity nor an increase in available carbon affected the ability of *S. putrefaciens* to metabolize pentavalent methylated arsenicals. Only high salt had an effect. These results suggest that *S. putrefaciens* may metabolize organoarsenicals differently in marine and terrestrial environments.

Organoarsenicals Uptake by *S. putrefaciens* 200

The ability to transform organoarsenicals requires that the substrates are transported into cells. To examine whether *S. putrefaciens* takes up organoarsenicals, cells were grown overnight in LB medium at 30 °C with shaking, washed and suspended at a cell density of $A_{600\text{ nm}} = 10$ in either ST 10^{-1} (low salt) or M9 (high salt) medium, and uptake of various organoarsenicals at 10 μM , final concentration, was determined. Although a high concentration of NaCl is required for organoarsenical biotransformations (Table 3), there was no significant difference in uptake of MAs(III), MAs(V) or DMAs(V) in *S. putrefaciens* in either M9 or ST 10^{-1} media (data not shown). Among the compounds assayed, MAs(III) was accumulated to the highest level. Figure 1 shows the results for cells in M9 medium, but the results were similar to cells suspended in ST 10^{-1} medium. Trivalent aromatic arsenicals were accumulated in lower amounts, while MAs(V) and DMAs(V) were accumulated at intermediate levels. Rox(V) was not taken up, which explains why there was no metabolism of Rox(V).

From those results it is clear that *S. putrefaciens* accumulated MAs(III) quite well. In those assays the cells had not been induced with arsenicals. In the *ars* gene cluster are two *arsP* genes, which potentially encode ArsP MAs(III) efflux permeases. *E. coli* cells expressing ArsP accumulated greatly reduced levels of MAs(III) compared with uninduced cells or with cells lacking an *arsP* gene, reflecting active efflux.¹⁴ Thus, in the absence of inducer, no ArsP activity would be expected. To examine the effect of induction of the *ars* genes, cells were induced with either As(III) or MAs(III), and uptake of inorganic or methylated species assayed. Compared with uninduced cells, the level of MAs(III) accumulation in cells induced with MAs(III) was reduced approximately 85% (Figure 2A), indicating that active extrusion of MAs(III) required induction by MAs(III). In contrast, As(III) did not induce MAs(III) extrusion, whereas cells induced with either MAs(III) or As(III) exhibited reduced uptake of inorganic As(III) (Figure 2B), which was catalyzed by ArsB.¹⁴ These results demonstrate that extrusion of inorganic arsenic and methylated arsenite were catalyzed by different transport pathways. MAs(III) extrusion was catalyzed by ArsP, which is specifically induced by MAs(III), while As(III) was extruded by ArsB, which was induced by either As(III) or MAs(III).

Environmental Implications

Reduction of Fe(III) and As(V) by metal-reducing bacteria is largely responsible for mobilization of environmental mineral-bound arsenic.³⁹ In regions of Bangladesh where there are extremely high levels of arsenic in groundwater, mobilization of As(V) is the result of bacteria such as *Shewanella* that have *arrAB* genes for the respiratory arsenate reductase.

Yet most current studies have focused on biotransformations of inorganic arsenic. The results of our study show that *S. putrefaciens* is surprisingly versatile in its ability to reduce, demethylate, and thiolate environmental organoarsenicals (Figure 3), including the herbicide MSMA and the animal husbandry growth promoter roxarsone. These data suggest that *S. putrefaciens* contributes to environmental release of inorganic arsenic by biotransformations of organoarsenicals that are either produced by soil microbes or introduced anthropogenically. We demonstrate here for the first time that organoarsenical thiolation occurs aerobically, which can contribute to mobilization and toxicity of organoarsenicals. While further studies are required for a detailed mechanistic understanding of organic arsenic biotransformation by the facultative anaerobe *S. putrefaciens*, it is likely that organoarsenical biotransformations by environmental microbes play a central role in the global arsenic biogeochemical cycle.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

MAs(III)	Methylarsenite
MAs(V)	methylarsenate
DMA(V)	dimethylarsenate
PhAs(III)	phenylarsenite
Rox(V)	roxarsone (3-nitro-4 hydroxybenzenearsonic acid)
Rox(III)	roxarsone with reduced As(III)
MSMA	monosodium methylarsenate
(HPLC)	high pressure liquid chromatography
(ICP-MS)	inductively coupled plasma mass spectroscopy
(MMMTAs(V))	monomethyl monothioarsonic acid
(DMMTAs(V))	dimethylmonothioarsonic acid
Nit(V)	Nitarsonic acid
(pAsA(V))	<i>p</i> -arsanilic acid
(3A4HBzAs(V))	3-amino-4-hydroxybenzenearsonic acid

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Figure 1.

Organoarsenic accumulation in LB-grown cells of *S. putrefaciens* incubated in M9 medium was assayed as described in Materials and Methods. Organoarsenicals were each added at 10 μ M, final concentration: (o), MAs(III); ($\square$), MAs(V); (∇), DMAs(V); ($\diamond$), Nit(III); ($\triangle$), Rox(III) and ($\blacktriangle$), Rox(V). Data are the mean $\pm$ SE ($n = 3$).

Figure 2.

Cultures of *S. putrefaciens* were cultured to the density of $A_{600} = 0.6$ at 30 °C with aeration in LB medium, washed and suspended at the same density in 5x-ST 10^{-1} medium supplemented with 0.2% glucose as carbon source, then induced with MAs(III) or As(III) at 2 μ M, final concentration, for 14 h 30 °C. Arsenic uptake of (A) MAs(III) or (B) As(III) at 10 μ M, final concentration, was assayed as described in Materials and Methods. (o), no induction; (∇), induced with As(III); ($\square$), induced with MAs(III). Data are the mean $\pm$ SE ($n = 3$).

Figure 3.

S. putrefaciens is a highly versatile soil and marine microbe that has multiple pathways of arsenic biotransformations. As(III), As(V) and Rox(III) are transported into cells of *S. putrefaciens* by unidentified carriers. As(V) is reduced either by the respiratory arsenate reductase for energy generation or by the ArsC resistance arsenate reductase. MAs(V) is reduced to MAs(III) by an unknown process, and both MAs(III) and MAs(V) are thiolated to MMMTAs-(V). The As(III) and nitro groups of Rox(III) are reduced, producing 3A4HBzAs-(V). The C-As bond in Rox(III) and MAs(III) are cleaved to release inorganic As(III), which is extruded from cells by the ArsB permease.

Table 1

Methylarsenical Biotransformations

		products found in culture medium (μM) ^a					
substrates	time (h)	As(III)	MAs(III)	MAs(V)	DMA _s (V)	DMMTA _s (V)	MMMTA _s (V)
MAs(III)	0	ND ^b	3.85 $\pm$ 0.15 (96.5 $\pm$ 3.8%) ^c	ND	ND	ND	ND
	4	1.18 $\pm$ 0.06 (29.6 $\pm$ 1.5%)	1.23 $\pm$ 0.08 (30.8 $\pm$ 2.0%)	0.38 $\pm$ 0.04 (9.5 $\pm$ 1.0%)	ND	ND	0.71 $\pm$ 0.05 (17.9 $\pm$ 1.3%)
	12	3.22 $\pm$ 0.13 (80.5 $\pm$ 3.3%)	ND	ND	ND	ND	0.45 $\pm$ 0.07 (11.3 $\pm$ 1.8%)
MAs(V)	0	ND	ND	3.91 $\pm$ 0.13 (97.8 $\pm$ 3.3%)	ND	ND	ND
	4	0.36 $\pm$ 0.03 (9.0 $\pm$ 0.8%)	0.83 $\pm$ 0.07 (20.8 $\pm$ 1.8%)	2.15 $\pm$ 0.11 (53.8 $\pm$ 2.8%)	ND	ND	0.23 $\pm$ 0.01 (5.8 $\pm$ 0.3%)
	12	3.10 $\pm$ 0.09 (77.5 $\pm$ 2.3%)	ND	ND	ND	ND	0.21 $\pm$ 0.01 (5.3 $\pm$ 0.3%)
DMA _s (V)	0	ND	ND	ND	3.93 $\pm$ 0.11 (98.3 $\pm$ 2.8%)	ND	ND
	4	ND	ND	ND	2.42 $\pm$ 0.08 (60.5 $\pm$ 2.0%)	1.14 $\pm$ 0.06 (28.5 $\pm$ 1.5%)	ND
	12	ND	ND	ND	0.23 $\pm$ 0.03 (5.7 $\pm$ 0.8%)	3.24 $\pm$ 0.13 (8.10 $\pm$ 3.3%)	ND

^a Transformations of methylarsenicals by *S. putrefaciens* were assayed in LB-grown cells incubated in M9 medium, as described in Materials and Methods. Cells were incubated with MAs(III), MAs(V), or DMA_s(V), each at 4 μM , final concentration. After 4 or 12 h, samples were assayed for arsenic biotransformations by HPLC using a C18 reverse phase column, and the amount of arsenic was estimated by ICP-MS. Data are the mean $\pm$ SE ($n = 3$).

^b ND: nondetectable.

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

Table 2

Aromatic Arsenical Biotransformations

		products found in culture medium (μM) ^d				
substrates	time (h)	As(III)	Rox(III)	Rox(V)	3A4HBzAs(V)	
Rox(III)	0	ND ^b	3.81 $\pm$ 0.19 (95.3 $\pm$ 4.8%) ^c	ND	ND	
	12	0.42 $\pm$ 0.03 (10.5 $\pm$ 0.8%)	1.17 $\pm$ 0.06 (29.3 $\pm$ 1.5%)	1.43 $\pm$ 0.09 (35.8 $\pm$ 2.3%)	0.59 $\pm$ 0.04 (14.8 $\pm$ 1.0%)	
Rox(V)	0	ND	ND	3.93 $\pm$ 0.11 (98.3 $\pm$ 2.8%)	ND	
	12	ND	ND	3.85 $\pm$ 0.17 (96.3 $\pm$ 4.2%)	ND	
		PhAs(III)	PhAs(V)	Unidentified ^d		
PhAs(III)	0	3.87 $\pm$ 0.15 (96.8 $\pm$ 3.8%)	ND	#1	#2	#3
	12	0.51 $\pm$ 0.04 (12.8 $\pm$ 1.0%)	0.34 $\pm$ 0.02 (8.50 $\pm$ 0.5%)	1.21 $\pm$ 0.07 (30.3 $\pm$ 1.8%)	0.46 $\pm$ 0.05 (11.5 $\pm$ 1.3%)	0.43 $\pm$ 0.08 (10.8 $\pm$ 2.0%)
PhAs(V)	0	ND	3.86 $\pm$ 0.16 (96.5 $\pm$ 4.0%)	ND	ND	ND
	12	ND	3.80 $\pm$ 0.23 (95.0 $\pm$ 5.8%)	ND	ND	ND
		As(III)	Nit(III)	Nit(V)	pAsA(V)	
Nit(III)	0	ND	3.84 $\pm$ 0.15 (96.0 $\pm$ 3.8%)	ND	ND	
	12	1.02 $\pm$ 0.05 (25.5 $\pm$ 1.3%)	ND	0.38 $\pm$ 0.04 (9.50 $\pm$ 1.0%)	2.04 $\pm$ 0.15 (51.0 $\pm$ 3.8%)	
Nit(V)	0	ND	3.87 $\pm$ 0.17 (96.8 $\pm$ 4.3%)	ND	ND	
	12	0.22 $\pm$ 0.03 (5.5 $\pm$ 0.8%)	ND	0.09 $\pm$ 0.02 (2.3 $\pm$ 0.5%)	3.14 $\pm$ 0.12 (78.5 $\pm$ 3.0%)	

^aTransformations of aromatic arsenicals by *S. putrefaciens* were assayed as described in Table 1. Cells were incubated with Rox(III) or Rox(V); PhAs(III) or PhAs(V); Nit(III) or Nit(V), each added 4 μM , final concentration. Samples were assayed for arsenic biotransformations by HPLC using a C4 reverse phase column and a C18 reverse phase column separately, and the amount of arsenic was estimated by ICP-MS. Data are the mean $\pm$ SE ($n = 3$).

^bND: nondetectable.

^cNumbers in parentheses are the percentage of added arsenic.

^dThree peaks of arsenic were observed that did not correspond to known standards.

Table 3

Biotransformation of MAs(V) and DMAs(V) Requires a High Salt Environment

medium	substrate	As(III)	products found in culture medium (μM) ^a				
			MAs(III)	MAs(V)	DMAs(V)	DMMTAs(V)	MMMTAs(V)
M9	MAs(III)	1.07 $\pm$ 0.08 (26.8 $\pm$ 2.0%) ^c	1.17 $\pm$ 0.11 (29.3 $\pm$ 2.8%)	0.79 $\pm$ 0.06 (19.8 $\pm$ 1.5%)	ND ^b	ND	0.56 $\pm$ 0.04 (14.0 $\pm$ 1.0%)
	MAs(V)	0.34 $\pm$ 0.03 (8.5 $\pm$ 0.8%)	0.93 $\pm$ 0.05 (23.3 $\pm$ 1.3%)	2.03 $\pm$ 0.12 (50.8 $\pm$ 3.0%)	ND	ND	0.27 $\pm$ 0.02 (6.8 $\pm$ 0.5%)
	DMAs(V)	ND	ND	ND	1.72 $\pm$ 0.10 (43.0 $\pm$ 2.5%)	1.89 $\pm$ 0.11 (47.3 $\pm$ 2.8%)	ND
ST	MAs(III)	1.02 $\pm$ 0.09 (25.5 $\pm$ 2.3%)	1.07 $\pm$ 0.10 (26.8 $\pm$ 2.5%)	0.80 $\pm$ 0.06 (20.1 $\pm$ 1.5%)	ND	ND	0.57 $\pm$ 0.04 (14.3 $\pm$ 1.0%)
	MAs(V)	ND	ND	3.82 $\pm$ 0.15 (95.5 $\pm$ 3.8%)	ND	ND	ND
	DMAs(V)	ND	ND	ND	3.77 $\pm$ 0.14 (94.2 $\pm$ 3.5%)	ND	ND

substrate	addition	As(III)	products found in culture medium (μM) ^a				
			MAs(III)	MAs(V)	DMAs(V)	DMMTAs(V)	MMMTAs(V)
MAs(V)	none	0.02 $\pm$ 0.01 (0.5 $\pm$ 0.3%) ^c	0.04 $\pm$ 0.02 (1.0 $\pm$ 0.5%)	3.41 $\pm$ 0.14 (85.3 $\pm$ 3.5%)	ND ^b	ND	ND
	NaCl	0.32 $\pm$ 0.04 (8.0 $\pm$ 1.0%)	0.90 $\pm$ 0.05 (22.5 $\pm$ 1.3%)	2.08 $\pm$ 0.10 (52.0 $\pm$ 2.5%)	ND	ND	0.25 $\pm$ 0.04 (6.3 $\pm$ 1.0%)
DMAs(V)	glucose	0.03 $\pm$ 0.02 (0.8 $\pm$ 0.5%)	0.06 $\pm$ 0.03 (1.5 $\pm$ 0.8%)	3.39 $\pm$ 0.15 (84.8 $\pm$ 3.8%)	ND	ND	ND
	none	ND	ND	ND	3.57 $\pm$ 0.11 (89.3 $\pm$ 2.8%)	0.08 $\pm$ 0.02 (2.0 $\pm$ 0.5%)	ND
	NaCl	ND	ND	ND	0.42 $\pm$ 0.03 (10.5 $\pm$ 0.8%)	3.19 $\pm$ 0.15 (79.8 $\pm$ 3.8%)	ND
	glucose	ND	ND	ND	3.52 $\pm$ 0.14 (88.0 $\pm$ 3.5%)	0.07 $\pm$ 0.01 (1.8 $\pm$ 0.3%)	ND

^aTop: Cells of *S. putrefaciens* were cultured overnight in LB medium, washed and suspended at a density of A600 of 3.0 in either M9 medium or ST 10^{-1} medium supplemented with 0.2% glucose as carbon source, then incubated with MAs(III), MAs(V) or DMAs(V), each at 4 μM final concentration, for 4 h 30 °C. Bottom: MAs(V) and DMAs(V) biotransformations were assayed cells incubated in ST 10^{-1} medium with or without added NaCl or glucose, as indicated, each at 0.1 M final concentration. Samples were separated by HPLC using a C18 reverse phase column, and the amount of arsenic in relative counts per second (cps) was estimated by ICP-MS. Data are the mean $\pm$ SE ($n = 3$).

ND: nondetectable.
Numbers in parentheses are the percentage of added arsenic.

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