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Demethylation of methylarsonic acid by a microbial community

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Summary

Arsenic is one of the most widespread environmental carcinogens and has created devastating human health problems worldwide, yet little is known about mechanisms of biotransformation in contaminated regions. Methylarsonic acid [MAs(V)], extensively utilized as an herbicide, is largely demethylated to more toxic inorganic arsenite, which causes environmental problems. To understand the process of demethylation of methylarsenicals, soil samples commonly used on Florida golf courses were studied. Several soil extracts were found to demethylate MAs(V) to inorganic arsenite [As(III)]. From these extracts, a bacterial isolate was capable of reducing MAs(V) to MAs(III) but not of demethylating to As(III). A second bacterial isolate was capable of demethylating MAs(III) to As(III) but not of reducing MAs(V). A mixed culture could carry out the complete process of reduction and demethylation, demonstrating that demethylation of MAs(V) to As(III) is a two-step process. Analysis of the 16S ribosomal DNA sequences of the two organisms identified the MAs(V)-reducing and the MAs(III)-demethylating isolates as belong to *Burkholderia* and *Streptomyces* species respectively. This is the first report of a novel pathway of degradation of a methylarsenical herbicide by sequential reduction and demethylation in a microbial soil community, which we propose plays a significant role in the arsenic biogeochemical cycle.

Introduction

Arsenic is the most prevalent toxic environmental toxin, entering the biosphere primarily from geochemical sources but also from anthropogenic activities such as the use of arsenical herbicides. The metalloid produces a wide range of health effects in humans, including cardiovascular and peripheral vascular diseases, diabetes mellitus and various forms of cancer (Abernathy *et al.*, 1999). As a consequence of its ubiquity and carcinogenicity,

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Supporting information

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Fig. S1. Phylogenetic relatedness of *Burkholderia* sp. MR1. A phylogenetic tree was constructed based on 16S rDNA sequences of the MAs(V) reducing strain *Burkholderia* sp. MR1 and 10 selected species from the genus *Burkholderia*. Bootstrap values calculated for 1000 subsets are indicated on each branch. The numbers in parentheses indicate accession numbers for each species, and the scale bar represents 1% sequence dissimilarity.

Fig. S2. Phylogenetic relatedness of *Streptomyces* sp. MD1. A phylogenetic tree was constructed based on 16S rDNA sequences of the MAs(III) demethylating strain *Streptomyces* sp. MD1 and 10 selected species from the genus *Streptomyces*. Bootstrap values calculated for 1000 subsets are indicated on each branch. The numbers in parentheses indicate accession numbers for each species, and the scale bar represents 1% sequence dissimilarity.

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arsenic ranks first on the US Environmental Protection Agency's (EPA) Superfund List of Hazardous Substances (<http://www.atsdr.cdc.gov/cercla/07list.html>).

Geothermal introduction of arsenic into the environment predates the origin of life, and its environmental ubiquity has provided a strong and persistent pressure for the evolution of detoxifying mechanisms (Bhattacharjee and Rosen, 2007; Rensing and Rosen, 2009). Most common are efflux systems that lower the concentration of intracellular arsenic (Rosen and Tamas, 2010). Alternate pathways for biotransformation of arsenic include oxidation of arsenite to arsenate, which can be coupled to energy production (Paez-Espino *et al.*, 2009) or methylation to produce a variety of organic species (Bentley and Chasteen, 2002). Both prokaryotic and eukaryotic microbes methylate arsenic by ArsM As(III) S-adenosylmethionine methyltransferases in an established detoxification process (Qin *et al.*, 2006; 2009). At the latest count, nearly 4000 ArsM genes have been deposited in the NCBI database. Most are of bacterial origin, but there are also a considerable number of fungal, algal and vertebrate homologues. The products of the methylation pathway include the less toxic pentavalent methylated arsenicals methylarsonic acid [MAs(V)], dimethylarsinic acid [DMAs(V) or cacodylate], trimethylarsine oxide [TMAsO(V)] (Challenger, 1951) and the non-toxic trivalent volatile trimethylarsine [TMAs(III)] (Cullen, 2005). Arsenic methylation has been proposed to play a significant role in the arsenic biogeochemical cycle among terrestrial, aquatic and atmospheric surroundings (Rensing and Rosen, 2009).

In addition to the widespread biological genesis of methylated arsenicals, salts of MAs(V) and DMAs(V) have been used as herbicides and insect pesticides. During the Vietnam War, DMAs(V), called Agent Blue, was used as a defoliant. At present 154 commercial products with these compounds are used as herbicides. Many of these are for weed control, especially for cotton, ornamental plants, lawns and golf course turf. Approximately 3 000 000 pounds of MAs(V) and 100 000 pounds of DMAs(V) are in commercial use in the USA. In 2006 the EPA denied eligibility for re-registration of MAs(V) and DMAs(V) as herbicides because the agency concluded that the benefits gained from their use did not outweigh the risk to humans (http://www.epa.gov/oppsrrd1/REDs/organic_arsenicals_red.pdf). In a follow-up in 2009, the EPA banned the use of organic arsenical herbicides after 31 December 2013 (EPA, 2009). The sole exception is for treatment of cotton because the EPA does not anticipate that arsenic in cotton will end up in the food supply.

As the EPA noted, however, arsenic, as an element, does not degrade and remains in soil for extended periods, and that the average aerobic half-life of MAs(V) in soil is 240 days. Its chemical form may change, influencing mobility and bioavailability. Soil organisms further methylate these organic arsenicals, including volatilization of the end product of the ArsM pathway, TMAs(III) (Qin *et al.*, 2006; 2009). A major concern of the EPA is that biotransformation between inorganic arsenicals and methylarsenicals is bidirectional, and that noncarcinogenic MAs(V) is demethylated to more toxic and carcinogenic inorganic arsenic through microbial transformations. Demethylation of methylarsenicals has been studied extensively in soil (Von Endt *et al.*, 1968; Woolson *et al.*, 1982; Akkari *et al.*, 1986; Gao and Buran, 1997; Feng *et al.*, 2005; Maki *et al.*, 2006a; Huang *et al.*, 2007), sludge (Sierra-Alvarez *et al.*, 2006), sediment (Hanaoka *et al.*, 1990), seawater (Sanders, 1979) and fresh water (Maki *et al.*, 2006b; 2009). Demethylating microbes have been found in soil (Von Endt *et al.*, 1968; Maki *et al.*, 2006a,b) and lake water (Maki *et al.*, 2004; 2006a). Only in a few cases have specific microorganisms been associated with demethylation such as *Mycobacterium neoaurum* (Lehr *et al.*, 2003), Montrachet wine yeast (Crecelius, 1977) and *Candida humicola* (Cullen *et al.*, 1979), and no molecular description of the demethylation pathway has been reported.

Even though the EPA has ordered its use to cease after 31 December 2013, MAs(V) is still commonly applied as an herbicide on golf courses in Florida. The run-off from such golf courses has contaminated the water supplies of Florida municipalities with arsenic (Pichler *et al.*, 2008; Whitmore *et al.*, 2008) and will remain a problem for decades to come. Water percolating through the soil of simulated golf course greens on which MAs(V) was applied is demethylated to As(V) (Feng *et al.*, 2005). In this study we show that demethylation of MAs(V) in these simulated golf course soils is the result of biological activity. No single microorganism isolated from those soils was capable of demethylating MAs(V). We identified a *Burkholderia* species capable of reducing MAs(V) to MAs(III) and a *Streptomyces* species that could demethylate MAs(III) to As(III). In mixed culture the two bacteria carried out the demethylation process from MAs(V) to As(III). These results illustrate a novel pathway for demethylation of an organic arsenical utilizing a communal sequential reduction and demethylation reactions catalysed by different soil microorganisms.

Results

MAs(V) demethylation by golf course soils

Previously different types of soil samples of varying porosities and organic content from simulated Florida golf course were shown to demethylate MAs(V) (Feng *et al.*, 2005). The ability of extracts from two of these soils, an uncoated sandy soil (S) and a soil consisting of uncoated sand and peat (S+P), to demethylate MAs(V) was examined. Extracts from both types of soil were prepared with ST 10⁻¹ medium (Maki *et al.*, 2004) and incubated with 1 μ M MAs(V) at room temperature for 1 week, following which the arsenic species in each culture were determined (Fig. 1A). Neither soil contained arsenic prior to addition of MAs(V), and no transformation of MAs(V) occurred following incubation in medium alone or in extracts of autoclaved soils. In contrast, nearly complete demethylation of MAs(V) to As(III) was observed in either S or S+P extracts. These results strongly indicate that MAs(V) demethylation is a biological process.

Although all of the MAs(V) was converted to As(III) by soil extracts, no single organism capable of demethylating MAs(V) was isolated. The possibility that MAs(V) reduction is an obligatory precursor to demethylation was examined. First, the fate of MAs(V) was examined after shorter incubations with the S+P soil extract (Fig. 1B). After 3 days of incubation, MAs(V) was reduced to MAs(III), with some As(III) production. After an additional 2 days, MAs(III) decreased with a corresponding increase in As(III) and, after a week, there was complete conversion to As(III). These results suggest a two-step pathway, with sequential reduction of MAs(V) to MAs(III), followed by demethylation of MAs(III) to As(III).

Isolation and identification of MAs(V)-reducing bacterium

Extracts of both S and S+P were screened for MAs(V)-demethylating organisms. Serial dilutions of the two soil extracts were applied to ST 10⁻¹ agar plates. Ten colonies from S extracts and 22 colonies from S+P extracts were screened for MAs(V) demethylating activity, but none exhibited ability to demethylate MAs(V).

In contrast, of 22 colonies from S+P that were unable to demethylate, eight exhibited ability to reduce MAs(V) to MAs(III) (a representative isolate is shown in Fig. 2). None of the isolates from extracts of S soil was able to reduce MAs(V). Analysis of 16S ribosomal DNA sequences from the eight colonies revealed that they all belong to the Gram-negative bacterium *Burkholderia* genus (accession number HM641907) (Fig. S1). Because of the selection conditions, the eight were probably derived from the same single isolate, so only

one, *Burkholderia* sp. MR1, was used for further analysis. Microscopic analysis revealed a rod shaped morphology consistent with *Burkholderia*.

Burkholderia are related to *Pseudomonas*, so we considered the possibility that the latter could also reduce MAs(V). *Pseudomonas putida* strain KT2440 (Herrero *et al.*, 1990) was found to reduce MAs(V) to MAs(III). The ability of *P. putida* strain KT2440 and *Burkholderia* sp. MR1 to reduce MAs(V) was compared in cells grown in Luria–Bertani (LB) broth, ST 10⁻¹ and M9 minimal medium supplemented with glucose as carbon source (Fig. 3). Neither organism exhibited reductase activity when grown in LB medium (Fig. 3B), and both could reduce when grown in either ST 10⁻¹ (Fig. 3A) or M9 basal salts media (Fig. 3C). ST 10⁻¹ is a nutrient-poor medium that better reflects environmental growth conditions than LB but limits total growth of the culture. For that reason, both organisms were grown overnight in LB, washed free of medium, suspended at equivalent cell densities in either ST 10⁻¹ or M9 and incubated at room temperature for the indicated times. Although reduction was better with *Burkholderia* sp. MR1, both organisms reduced MAs(V) in either ST 10⁻¹ (Fig. 3D) or M9 (Fig. 3F) but not LB (Fig. 3E), suggesting that the effect of growth in LB medium is reversible. A possibility for the lack of reduction in cells grown in LB is that uptake of MAs(V) is lower in those cells. However, there was no significant difference in uptake in *P. putida* strain KT2440 and *Burkholderia* sp. MR1, whether grown in LB or ST 10⁻¹ media (Fig. 4). This suggests that an unidentified component of LB medium may repress or inhibit reduction.

Pseudomonas putida strain KT2440 could only reduce MAs(V) to MAs(III) but could not demethylate MAs(III), unlike the environmental *P. putida* isolates reported to be able to demethylate MAs(V) (Maki *et al.*, 2006a), which might have acquired additional genes. The *P. putida* genome has two *arsRBCH* operons (Canovas *et al.*, 2003). In addition, three additional genes following the *arsI* operon putatively encode a phosphatase (accession number NP_744079.1), a monooxygenase (accession number NP_744078.1) and a phosphinothricin *N*-acetyltransferase (accession number NP_744077.1). The monooxygenase and the phosphinothricin *N*-acetyltransferase possess around 45% similarity to the NADH : flavin oxidoreductase and the GCN5-related *N*-acetyltransferase, respectively, in *ars* operons of *B. ambifaria* AMMD. The functions of these putative proteins in arsenic metabolism are unknown, so it is possible that they contribute to MAs(V) reductase activity. Strains *P. putida* TEC1 (Galvao and de Lorenzo, 2005), a derivative of KT2440, and a *arsI&2* strain derived from TEC1 by deletion of the two *ars* operons (gift from V. de Lorenzo) were compared with *Burkholderia* sp. MR1 and *Escherichia coli* W3110 (Fig. 5). Each *P. putida* strain exhibited MAs(V) reductase activity, demonstrating that the activity is not catalysed by a product of either of the *ars* operons. In contrast, *E. coli* did not reduce the pentavalent arsenical. No significant difference was observed, which indicates that MAs(V) metabolism is not catalysed by *ars* operon genes, at least in *P. putida* KT2440. These results show that both *Burkholderia* and *Pseudomonas* but not *E. coli* have enzymatic mechanisms for MAs(V) reduction, and that this activity is most likely not encoded by traditional *ars* operons.

Isolation and identification of MAs(III)-demethylating bacterium

Isolates were screened for the ability to demethylate MAs(III). Individual isolates were incubated with MAs(V) in filter-sterilized S+P extract, either alone or in co-culture with *Burkholderia* sp. MR1. One isolate capable of demethylation, shown to be a *Streptomyces* species, was identified (Fig. 6A). By itself, this isolate, *Streptomyces* sp. MD1, produced only small amounts of As(III) (Fig. 6A, curve 3), but, when co-cultured with *Burkholderia* sp. MR1, achieved complete transformation of MAs(V) to As(III) (Fig. 6A, curve 4). Since the *Burkholderia* species only reduces MAs(V) to MAs(III), these results suggest that *Streptomyces* sp. MD1 catalyses demethylation of MAs(III) to As(III). This was confirmed

by demonstrating that *Streptomyces* sp. MD1, incubated with chemically prepared MAs(III) in the absence of *Burkholderia* sp. MR1, produced As(III) (Fig. 6B, curve 3). Interestingly, in this experiment much of the MAs(III) spontaneously oxidized to MAs(V) (Fig. 6B, curves 2 and 3), but in co-culture no oxidation was observed (Fig. 6A, curve 4). Analysis of the 16S rDNA sequence (accession number HM641908) from this isolate identified it as a Gram-positive streptomyces (Fig. S2). The cells had a filamentous morphology consistent with that of *Streptomyces*. Unlike MAs(V) reduction, MAs(III) demethylation was observed with *Streptomyces* sp. MD1 grown in LB medium, as well as M9 or ST 10⁻¹ medium (Fig. 7), indicating that this activity is constitutive.

Discussion

Given the widespread occurrence of arsenic in water and soil, microbial transformation into methylated species takes place continuously worldwide. Although difficult to quantify, we consider it likely that global microbial production of methylarsenicals far exceeds anthropogenic introduction. The major product of the ArsM reaction is DMAs(V), and the final product, which is formed rather slowly, is non-toxic TMAs(III) gas (Cullen, 2005). If this is indeed a pathway for arsenic detoxification, why do microorganisms reduce MAs(V) to the more toxic MAs(III) and then demethylate it to As(III), which is also more toxic than MAs(V)? The most likely explanation is that aerobically As(III) is oxidized to less toxic As(V). Of course, other organisms constantly reduce and methylate As(V) again, which continues the global arsenic cycle. Other possibilities are that MAs(V) reduction produces energy analogous to the role of respiratory arsenate reductases in anaerobes and that demethylation allows for utilization of the methyl groups for biomass. These are unlikely to be assimilatory pathways because the amount of MAs(V) even in highly contaminated soils would not be sufficient for utilization as a primary energy or carbon source, although it could supplement other supplies. In addition, *Burkholderia* and *Pseudomonas* are aerobes that do not have *arrAB* genes that encode the subunits of the respiratory arsenate reductase.

Regardless of the driving force for evolution of demethylation pathways for MAs(V), the ability has apparently been acquired multiple times by multiple organisms, perhaps as adventitious reactions by pathways with other functions. In some studies single organisms have been reported to carry out conversion of MAs(V) to As(III). For example, Lehr *et al.* (2003) isolated a strain of *Mycobacterium neoaurum* that demethylates both MAs(V) and MAs(III). Maki *et al.* (2006a) identified several strains of *P. pudita* closely related to strain KT2440 from contaminated sites that could demethylate MAs(V). Although the *P. pudita* strains examined in this study do not demethylate, environmental isolates may have acquired the requisite genes through horizontal transfer.

In this study we observed that cultures from golf course soil treated with MAs(V) exhibited MAs(V) demethylation as the result of biological activity but were unable to isolate single organisms that could carry out the entire process. Instead, the entire demethylation process required communal action of at least two microbes, reduction by *Burkholderia* sp. MR1 (or by *Pseudomonas* sp.) and demethylation by *Streptomyces* sp. MR1. Many *Burkholderia* are soil organisms, plant pathogens and/or agriculturally important species. The genome of the related *Burkholderia ambifaria* AMMD (Fig. 3) has two *ars* operons and additional non-operon *ars* genes in Chromosome 1 that include *arsR* genes for As(III)-responsive transcriptional repressors, *arsB* genes for arsenite efflux carriers, *arsC* genes for arsenate reductases, an *arsH* gene for an NADPH-FMN oxidoreductase and several genes of unknown function, including a putative NADH: flavin oxidoreductase (accession number ABI89703.1) and a putative GCN5-related acetyltransferase genes (accession number ABI89704.1). It is not known whether any of the gene products of the *Burkholderia* *ars* operons can reduce MAs(V). The genome contains neither genes for respiratory arsenate

reductases or arsenite oxidases nor an *arsM* gene for an As(III)-S-adenosylmethionine methyltransferase.

Why would *Burkholderia* sp. MR1 (or *P. pudita* KT2440) reduce MAs(V) to the more toxic MAs(III)? One explanation is that known *Burkholderia* and *Pseudomonas* genomes do not have *arsM* genes, so cannot further methylate MAs(V) to TMAs(III) gas. These soil organisms take up MAs(V), reduce it to MAs(III), which is then extruded into the medium (Fig. 8). By removing MAs(III) from the cytosol, the cells reduce toxicity to themselves (but not to other organism). Even so, this is probably an adventitious process, especially considering that a *P. pudita* strain lacking *ars* operons can still reduce MAs(III). How does MAs(V) get into these bacteria? Perhaps MAs(V) is taken up by phosphate permeases, the route of As(V) uptake into most cells (Rosen and Tamas, 2010). MAs(V) can also be taken up by aquaglyceroporins (AQPs) at low pH as the fully protonated species (Li *et al.*, 2009; McDermott *et al.*, 2010), and both *Burkholderia* and *Pseudomonas* have genes encoding AQPs. Or perhaps there are other unidentified transporters that adventitiously take up the pentavalent species. The process of MAs(V) reduction to MAs(III) is also unknown, and identification of the responsible gene(s) is essential. Both organisms have *arsC* genes for As(V) reduction, but *ArsC* does not reduce MAs(V). In addition, *P. pudita* derivatives lacking *ars* operons reduce MAs(V), demonstrating that MAs(III) is reduced, perhaps adventitiously, by the product of other genes. Once produced, how does the intracellular MAs(III) get out of cells? Both organisms have genes for Acr3 and/or ArsB, the major arsenite extrusion proteins in bacteria (Rosen and Tamas, 2010). It is not known whether these proteins can also catalyze extrusion of MAs(III). AQPs, however, facilitate MAs(III) translocation (Liu *et al.*, 2004). As long as the MAs(III) is produced internally, efflux will always be downhill, allowing a channel such as an AQP to lower the intracellular concentration. In an analogous situation, *Sinorhizobium meliloti* detoxifies As(V) by internal reduction to As(III) followed by downhill efflux via AqpS (Yang *et al.*, 2005).

As MAs(III) appears, other organism are challenged to detoxify it. Note that, as MAs(III) is re-oxidized to MAs(V), *Burkholderia* continuously reduces it back to MAs(III) (Fig. 6A, curve 4), so other microbes must have evolved MAs(III) detoxification pathways to deal with the build-up of MAs(III). Considering that As(III) is less toxic than MAs(III), demethylation of MAs(III) to As(III) by *Streptomyces* might be a detoxification process. It is interesting to note that groundwater from a former herbicide production plant on the Menominee River in Wisconsin was shown to contain high concentrations of MAs(III) (McKnight-Whitford *et al.*, 2010), which suggests that first microbial reduction of MAs(V) may be widespread and second that MAs(III) is indeed a problem even with an oxidizing atmosphere. Following demethylation, some microorganisms can re-methylate As(III), but, again, the genome of the closely related *Streptomyces coelicolor* does not have an *arsM* gene. *Streptomyces* sp. MR1 can take up MAs(III), perhaps via AQPs or by glucose permeases, which also transport MAs(III) (Liu *et al.*, 2006; Jiang *et al.*, 2010). Inside the cells, MAs(III) is demethylated to As(III), again by unknown mechanisms. It is not known whether the methyl group is incorporated into biomass or simply evolves it as CO₂. Characterization of this pathway is also essential to more completely understand environmental arsenic cycling. Finally, *Streptomyces* has both *arsB* and *acr3* genes, so demethylation to As(III) allows for extrusion via one or more of the arsenite efflux systems, where it can be re-oxidized to As(V) and remethylated, continuing the arsenic biogeochemical cycle.

Experimental procedures

Chemicals

The commercial herbicide Ortho Weed-B-Gone Crabgrass Killer Formula II, which contains calcium methanearsonate as its sole active ingredient, was used as the source of

monomethylarsonic acid [MAs(V)]. The concentration of stock solutions of MAs(V) was determined by ICP-MS (ELAN 9000, PerkinElmer, Waltham, MA, USA). Unless otherwise mentioned, all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Strains

The *P. putida* strains used in this study KT2440 (Franklin *et al.*, 1981), Tec1 (Galvao and de Lorenzo, 2005) and a double *ars* operon strain *ArsI&2* derived from Tec1 were kindly supplied by Víctor de Lorenzo, Centro Nacional de Biotecnología-CSIC, Madrid, Spain. *Escherichia coli* strain W3110 (Bachmann, 1987) was also used.

Demethylation of MAs(V) by soil extracts

Two types of soil samples from simulated golf course greens, including uncoated sand (S) and uncoated sand and peat (S+P), were examined (Feng *et al.*, 2005). To prepare soil extracts, native or autoclaved soils were mixed with ST 10⁻¹ culture medium (0.5 g l⁻¹ Difco Bacto Peptone and 0.05 g l⁻¹ yeast extract (Becton, Dickinson and Company) (Maki *et al.*, 2006b) in a 1:1 ratio (g:ml) and incubated for 2 h with moderate shaking. After centrifugation at 500 r.p.m. for 1 min to remove the soil, each of the resulting supernatant solutions was incubated with 1 µM MAs(V) with shaking at 200 r.p.m. at room temperature for the indicated lengths of time. The cultures were then centrifuged at 13 000 r.p.m. for 10 min to remove cells, and the resulting supernatants were filtered with a Microcon Ultracel YM-3 centrifugal filter (Millipore Corp, Billerica, MA, USA). Arsenic species in the filtered samples were analysed with a Series 2000 HPLC connected to an ELAN 9000 ICP-MS (Perkin Elmer, Waltham, MA, USA) using a Jupiter 5µ C18 300A reverse-phase C18 column (Chromservis s.r.o., Brno, Czech Republic), as described previously (Qin *et al.*, 2006).

Isolation of MAs(V) reducing and MAs(III) demethylating microorganisms

S+P or S soil extracts were diluted and spread on ST 10⁻¹ plates containing 1 µM MAs(V). The plates were incubated for 3 days at room temperature. Single colonies were purified by re-streaking, isolated and incubated in ST 10⁻¹ medium containing 1 µM MAs(V) at room temperature with shaking at 200 r.p.m. To examine the morphology of the isolated strains, the cells were fixed, stained with 1% methylene blue solution and observed by visible microscopy. After 3 days, transformation of MAs(V) was analysed by HPLC-ICP-MS, as described above. Although no single isolate was capable of demethylating MAs(V), a *Burkholderia* sp. isolate designated *Burkholderia* sp. MR1 [MAs(V) reducing] (*vide infra*) reduced MAs(V) to MAs(III). To further screen for MAs(III)-demethylating organisms, S+P soil extracts were filter-sterilized using a 0.2 µm Corning syringe filter and inoculated with the MAs(V)-reducing bacterium *Burkholderia* sp. or other isolates to reduce MAs(V) to MAs(III).

One isolate of *Streptomyces* sp. called *Streptomyces* sp. MD1 (MAs(III) demethylating) (*vide infra*) was identified through this second round of screening. This isolate was incubated in filter sterilized S+P extract containing with 1 µM MAs(V) in the presence or absence of the *Burkholderia* isolate for 4 days at room temperature, following which MAs(V) transformation products were analysed by HPLC-ICP-MS. The MAs(III)-demethylating activity was confirmed by incubating the *Streptomyces* isolate with MAs(III) prepared from MAs(V) by reduction with metabisulfite/thiosulfate (Reay and Asher, 1977). This chemically prepared MAs(III) reagent was adjusted to pH 6.5 and analysed by HPLC-ICP-MS. MAs(III) was added to filter sterilized S+P extract at 1 µM, final concentration, and incubated with or without the *Streptomyces* sp. isolate for the indicated times, following which transformation of MAs(III) was analysed by HPLC-ICP-MS.

MA(V) reduction and MA(III) demethylation in selected growth media

To analyse the effect of growth medium on MA(V) reductase activity of *Burkholderia* sp. MR1 and *P. putida* KT2440, each was separately cultured with 1 μ M MA(V) in ST 10⁻¹, LB (Sambrook *et al.*, 1989) or M9 medium (Sambrook *et al.*, 1989) supplemented with 0.2% D-glucose as carbon source, at room temperature with shaking (200 r.p.m.) for 3 days. To examine the reversibility of inhibition of MA(V) reduction by LB, *Burkholderia* sp. MR1 and *P. putida* KT2440 were cultured in LB overnight. After washing the cells once with either ST 10⁻¹, LB or M9 medium supplemented with 0.2% D-glucose, the cell density was adjusted to $A_{600} = 3.0$, transferred to the same medium and incubated with 1 μ M MA(V) at room temperature with shaking (200 r.p.m.) for 6 h. To examine the effect of growth media on MA(III)-demethylating activity, *Streptomyces* sp. MD1 was cultured in LB medium overnight at room temperature, washed once with ST 10⁻¹, LB or M9 medium supplemented with 0.2% D-glucose, transferred into the same volume of the respective media and incubated with 1 μ M MA(III) at room temperature with shaking (200 r.p.m.) for 3 h. MA(V) transformation in each culture was analysed by HPLC-ICP-MS.

MA(V) reduction by *Pseudomonas putida* KT2440 derivatives

MA(V) reductase activity of *P. putida* strains was analysed and compared with that of *Burkholderia* sp. MR1 and *E. coli* strain W3110. Each strain was separately incubated with fresh 1 μ M MA(V) in ST 10⁻¹ medium at RT with shaking (200 r.p.m.). Cultures of *P. putida* Tec1 and *Ars1&2* were supplemented with 20 μ g ml⁻¹ uracil. After 3 days, MA(V) transformation in each culture was analysed by HPLC-ICP-MS.

MA(V) uptake by *Burkholderia* sp. MR1 and *P. putida* KT2440

Cultures (100 ml) of *Burkholderia* sp. MR1 and *P. putida* strains of KT2440 were grown to $A_{600} = 1$ in LB medium. The cells were collected, washed once and suspended in either 10⁻¹ or LB medium at a density of $A_{600} = 10$. MA(V) was added to 1 ml of the prepared cell solutions at a final concentration of 1 mM to initiate the transport reaction. Portions (0.1 ml) were withdrawn at the indicated times, filtered through 0.45 mm pore size nitrocellulose filters (Millipore), washed twice with 4 ml of the same medium and air dried. The filters were digested with 0.3 ml of 70% HNO₃ at 70°C for 30 min, allowed to cool to room temperature and diluted with 5.7 ml of deionized water to produce a final concentration of HNO₃ of 3.5%. Arsenic in the prepared samples was quantified by ICP-MS. Arsenic solutions in the range of 1–50 p.p.b. were prepared in 3.5% HNO₃ using an arsenic standard (Ricca Chemical Company, Arlington, TX, USA).

16S rDNA sequence analysis

Total DNA from isolates was purified following cetyltrimethylammonium bromide extraction (Wilson, 2001). For the MA(III)-demethylating isolate it was first necessary to treat with lysozyme before CTAB extraction (Zehner *et al.*, 2005). Primers were designed from the highly conserved regions of the bacterial 16S ribosomal DNA (Lane *et al.*, 1985; Weisburg *et al.*, 1991). The sequences of two sense oligonucleotides, f27 and f2L, were, respectively, 5'-AGAGTTTGATCCTG GCTCAG-3' and 5'-CCAGCAGCCGCGGTAATAC-3' and those of the two antisense oligonucleotides, 1492r and r3L, were, respectively, 5'-GGCTACCTTGTTACGACTT-3' and 5'-TTGCGCTCGTTGCGGGACT-3'. To amplify 16S rDNA, polymerase chain reaction (PCR) was performed for each of the extracted total DNA with an annealing temperature of 61°C using Pfu Turbo DNA polymerase (STRATAGENE) and primer set of 27f and 1492r. Each PCR product was gel-purified using a QIAquick Gel Extraction Kit (QIAGEN Sciences) and then incubated with dATP, MgCl₂ and Taq polymerase (Invitrogen) at 75°C for 1 h to attach dATP to the 3' end. Each dATP-attached fragment was TA-cloned into

pGEM-T easy vector (Promega). T7 (sense) and SP6 (antisense) primers 5'-TAATACGACTCACTATAGGG-3' and 5'-ATTTAGGTGACACTATAGAA-3', respectively, were designed from the pGEM-T easy vector. Sequencing reactions for each cloned 16S rDNA were performed with f27, f2L, 1492r, r3L, T7 or SP6 primers, and each sequence was analysed using a CEQ 2000XL DNA Analysis System (Beckman Coulter). The partial sequence obtained for each 16S rDNA was searched with NCBI BLAST to determine species of each isolate. The GenBank accession numbers for the 16S rDNA sequences of *Burkholderia* sp. MR1 and *Streptomyces* sp. MD1 are HM641907 and HM641908 respectively. For phylogenetic analyses, each 16S rDNA sequence was aligned with those of 10 randomly selected strains from the same genus available in the DDBJ/EMBL/GenBank databases using CLUSTAL W version 1.83 (Thompson *et al.*, 1994) at the DDBJ web site (<http://clustalw.ddbj.nig.ac.jp/top-j.html>). Phylogenetic tree for each genus was constructed by neighbour-joining (Saitou and Nei, 1987), with distance calculation by a two-parameter method (Kimura, 1980) and drawn with TreeView (Page, 1996).

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Fig. 1.

Demethylation of MAs(V) to As(III) by soil extracts.

A. Transformation of MAs(V) by soil extracts was analysed after 1 week incubation at room temperature by reverse-phase HPLC-ICP-MS, as described under *Experimental procedures*, where the *x*-axis represents retention time, and the *y*-axis represents relative amounts of arsenic expressed as counts per second (cps). Curve 1: 1 μ M MAs(V) in ST 10^{-1} medium. Curve 2: S+P extracted in ST 10^{-1} medium. Curve 3: autoclaved S+P extracted in ST 10^{-1} medium containing 1 μ M MAs(V). Curve 4: S extracted in ST 10^{-1} medium containing 1 μ M MAs(V). Curve 5: S+P extracted in ST 10^{-1} medium containing 1 μ M MAs(V). B. The transformation of 1 μ M MAs(V) by S+P soil extracted in ST 10^{-1} medium was assayed after

incubation for 3 (curve 2), 5 (curve 3) and 7 (curve 4) days at room temperature. Curve 1: 1 μ M MAs(V) control incubated in ST 10^{-1} medium for 7 days.

Fig. 2. Reduction of MAs(V) by *Burkholderia* sp. MR1. Reduction of MAs(V) by a bacterial isolate identified as *Burkholderia* sp. MR1 was assayed by reverse-phase HPLC-ICP-MS, as described under *Experimental procedures*. Curve 1: 1 μ M MAs(V) in ST 10^{-1} medium. Curve 2: cells of *Burkholderia* sp. MR1 in ST 10^{-1} medium containing 1 μ M MAs(V).

Fig. 3.

Effect of growth media on MAS(V) reductase activity of *Burkholderia* sp. MR1 and *P. putida* KT2440.

A–C. Each bacterial strain was inoculated into ST 10^{-1} medium (A), LB medium (B) or M9 medium supplemented with 0.2% glucose as carbon source (C), and incubated with 1 μ M MAS(V) for 3 days at room temperature, following which reduction was analysed by reverse-phase HPCL-ICP-MS.

D–F. Each strain was cultured overnight in LB medium, washed and suspended at a density of A_{600} of 3.0 in ST 10^{-1} medium (A), LB medium (B) or M9 medium supplemented with 0.2% glucose as carbon source (C), and then incubated with 1 μ M MAS(V) at room temperature for 6 h. In each panel curve 1 shows medium with no cells; curve 2 shows *P. putida* KT2440 and curve 3 shows *Burkholderia* sp. MR1.

Fig. 4.

Uptake of MAS(V). The ability of *Burkholderia* sp. MR1 and *P. putida* KT2440 to take up MAS(V) was assayed as described under *Experimental procedures*. (●), *Burkholderia* sp. MR1 in ST 10⁻¹ medium; (○), *Burkholderia* sp. MR1 in LB medium; (△), *P. putida* KT2440 in ST 10⁻¹ medium; (▼), *P. putida* KT2440 in LB medium. The error bars represent the standard deviation of three assays.

Fig. 5.

The *P. putida* *ars* operon does not encode a MAs(V) reductase. MAs(V) reduction was assayed in the indicated bacterial cultures following growth in ST 10^{-1} medium containing 1 μ M MAs(V) for 3 days at room temperature. Curve 1: 1 μ M MAs(V) control in ST 10^{-1} medium; curve 2: *E. coli* strain W3110; curve 3: *Burkholderia* sp. MR1; curve 4: *P. putida* strain Tec1; curve 5: *P. putida* strain *Ars1&2*.

Fig. 6. Demethylation of MAs(V) by *Streptomyces* sp. MD1. Demethylation of MAs(III) by a bacterial isolate identified as *Streptomyces* sp. MD1 was assayed by reverse-phase HPLC-ICP-MS, as described under *Experimental procedures*.
 A. Demethylation of MAs(V) in mixed culture. Strains were incubated separately or together in filter-sterilized S+P extract containing 1 μ M MAs(V) for 1 week at room temperature. Curve 1: 1 μ M MAs(V) in filter-sterilized S+P. Curve 2: *Burkholderia* sp. MR1. Curve 3: *Streptomyces* sp. MD1. Curve 4: co-culture of *Burkholderia* sp. MR1 and *Streptomyces* sp. MD1.

B. Demethylation of MAs(III) by *Streptomyces* sp. MD1. MAs(III) was prepared by chemical reduction of MAs(V), as described under *Experimental procedures*. Line 1: MAs(III) in filter sterilized S+P extract without incubation. Line 2: MAs(III) in filter-sterilized S+P extract after three days of incubation at room temperature. Line 3: *Streptomyces* sp. MD1 incubated with MAs(III) for 3 days at room temperature.

Fig. 7. Effect of growth media on MAs(III) demethylating activity of *Streptomyces* sp. MD1. *Streptomyces* sp. MD1 was grown overnight in LB medium at 37°C, washed and suspended the same volume in ST 10⁻¹ medium (A), LB medium (B) or M9 medium supplemented with 0.2% glucose as carbon source (C), and incubated with 1 μM MAs(III) for 3 h at room temperature, following which demethylation was analysed by reverse-phase HPCL-ICP-MS. In each panel curve 1 shows 1 μM MAs(III) in medium with no cells and no incubation; curve 2 shows 1 μM MAs(III) in medium with no cells after 3 h; and curve 3 shows *Streptomyces* sp. MD1 incubated for 3 h in medium containing 1 μM MAs(III).

Fig. 8. Demethylation of MAs(V) is a two-step process carried out by a microbial community. Demethylation of MAs(V) in soil takes place by sequential reduction to MAs(III) by *Burkholderia* sp. MR1 followed by demethylation of MAs(III) to As(III) by *Streptomyces* sp. MD1. *Burkholderia* sp. MR1 takes up MAs(V) by unknown transporters (a), possibly phosphate transporters or AQPs, and extrudes MAs(III) by efflux systems (b), possibly ArsB, Acr3 or AQPs. *Streptomyces* sp. MD1 takes up MAs(III), possibly by AQPs or glucose permeases (c), and extrudes As(III) by ArsB or Acr3 (d).