Engineering the soil bacterium *Pseudomonas putida* for arsenic methylation

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Abbreviations: Methylarsenite, MAs(III); methylarsenate, MAs(V); dimethylarsenate, DMA(s)(V); trimethylarsine oxide, TMAs(V)O; trimethylarsine, TMAs(III); S-adenosylmethionine, SAM; polymerase chain reaction, PCR; Luria-Bertani, LB; sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE); high pressure liquid chromatography (HPLC); inductively coupled plasma mass spectroscopy (ICP-MS).
Summary

Accumulation of arsenic has potential health risks through consumption in food. Here we inserted the gene As(III) S-adenosylmethionine methyltransferase (ArsM) into the chromosome of *Pseudomonas putida* KT2440. Recombinant bacteria methylate inorganic arsenic into less toxic organoarsenicals. This has potential for bioremediation of environmental arsenic and reducing arsenic contamination in food.
Arsenic is a Class I human carcinogen that poses a health risk to humans. Arsenic exposure is linked to skin cancer, bladder cancer, diabetes, cardiovascular disease and peripheral vascular disease (1, 2). The U.S. Environmental Protection Agency (EPA) ranks arsenic first its Superfund List of Hazardous Substances (http://www.atsdr.cdc.gov/SPL/index.html).

Arsenic is released into the environment by geothermal activity, from dissolution of minerals and from anthropogenic activities such industrial effluents, combustion of fossil fuels and the use of arsenic-containing pesticides, herbicides, wood preservatives and feed additives (3). As a result of the use of arsenic-contaminated irrigation water, arsenic accumulates in rice, the dietary staple for half the world’s population (4). Arsenic methylation is a detoxification pathway (5, 6). Many organisms have genes that encode As(III) S-adenosylmethionine (SAM) methyltransferases (termed ArsM in microbes, AS3MT in higher organisms) that biotransform As(III) to methylated species, with volatile nontoxic trimethylarsine (TMAs(III)) (7) as end product (5, 6, 8, 9). Pseudomonas putida KT2440 is a gram-negative bacterium found in water and soil, particularly in the rhizosphere at a relatively high population density (10). This soil microorganism has been studied extensively as a model for biodegradation of aromatic compounds such as naphthalene (11) and polystyrene (12, 13). Conventional remediation methods such as soil excavation followed by coagulation-filtration or ion exchange are expensive, disruptive and not widely used (14). Sphingomonas desiccabilis and Bacillus idriensis expressing arsM can remove arsenic from contaminated soil, but expression from a plasmid limits their utility (15). Pseudomonas species have prospect of rhizoremediation of organic compounds (16) but have not been
used for arsenic removal.

The objective of this study was to construct a strain of *P. putida* KT2440 with the potential for removal of arsenic from contaminated soil. We used the *Chlamydomonas reinhardtii* *arsM* gene encoding an ArsM orthologue (CrArsM). *In vitro*, purified CrArsM methylated As(III) to a variety of species (supplemental Fig. 1S_A). After 7 hrs, MAs(III) and DMAs(V) were produced in relatively equal amounts. After 14 hrs, the product was primarily DMAs(V), with lesser amounts of TMAs(V)O and no MAs(III). These results are consistent with sequential methylation steps to the mono-, di- and trimethyl products. TMAs(III) gas could be detected on H$_2$O$_2$-impregnated filters by oxidation to TMAs(V)O (supplemental Fig. 1S_B). These results demonstrate that purified CrArsM catalyzes three sequential rounds of As(III) methylation and converts toxic inorganic arsenic to less toxic or non-toxic organic arsenicals.

The *C. reinhardtii* *arsM* gene behind the kanamycin promoter was integrated into the chromosome of *P. putida* KT2440, which does not have an *arsM* gene and does not methylate arsenic. Mini-transposon pBAM1 was used as a suicide vector to generate stable integrants that could express *arsM* constitutively. The *arsM* gene was cloned into pBAM1 (Supplemental Fig. 2S) and subsequently transferred from *E. coli* CC118$\lambda$pir to *P. putida* KT2440 by tripartite conjugation with a helper strain. Wild type *P. putida* has two chromosomal *arsRBCH* operons and can grow in the present of 2 mM As(III), which provides a competitive advantage to *P. putida* in contaminated soil. (10). This could be crucial factor for sustaining growth of cells in the presence of indigenous bacterial populations (14). Cells of *P. putida* KT2440 expressing CrArsM were resistant to 7.5 to 10 mM As(III) in liquid basal salts.
M9 medium (Fig. 1). Biotransformation of arsenic by the cells was assayed with 25 μM As(III) or As(V) (Fig. 2). After 12 hrs, engineered *P. putida* biomethylated As(III) primarily to DMAs(V), and, to a lesser degree, MAs(V) (Fig. 2A). In a time-dependent fashion, the engineered cells produced DMA(III)H and TMAs(III) gases, identified by oxidizing them to DMA(V) and TMAs(V)O with H₂O₂ (Fig. 2B). In addition, the product of the methylation reaction was quantified in cells of *P. putida* expressing CrArsM. After 48 hrs, the major product found in the culture medium was DMA(V) (57% of total arsenic), with lesser amounts of MAs(V) (31%) and even less TMAs(V)O (8%) (Fig. 2C). The transgenic *P. putida* strain rapidly methylated As(V) (Fig. 2A, curve 4). There are two *arsRCBH* operons in the chromosome of *P. putida*, so it is reasonable to assume the chromosomally encoded ArsC reductase rapidly reduced As(V) to As(III), the substrate of CrArsM, allowing the soil bacterium to methylate both As(V) and As(III).

Under comparable conditions, cells of *E. coli* BL21(DE3) expressing *arsM* produced mainly DMA(V) (Fig. 1S_A), while *P. putida* expressing *arsM* produced significant amounts of MAs(V) (Fig. 2A,C). The monomethyl species is the precursor of the dimethyl species (17), indicating that the rate of methylation in *E. coli* is greater than *P. putida*. The amount of CrArsM produced by cells of both species expressing *arsM* was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by quantitative Western blotting using an antibody to the six-histidine tag on CrArsM (Supplemental Fig. 4S). More enzyme is produced in *E. coli*, most likely because more CrArsM is produced from a plasmid behind the strong T7 promoter compared with the chromosomally-encoded gene in *P. putida*. Yet, lower expression in *P. putida* may be advantageous under environmental conditions,
where overproduction of a heterologous protein can be detrimental to in situ performance (18). *P. putida* expressing *arsM* is 5-fold more resistant to arsenite than the wild type strain, which gives it the ability to grow in arsenic-contaminated soils. Within 16 hrs the cells transformed nearly all of the inorganic arsenic in the culture medium to the less toxic methylated species, including methylarsenate (MAs(V)), DMAs(V), trimethylarsine oxide (TMAs(V)O) and eventually TMAs(III) gas, which further reduces the content of inorganic arsenic in soil and surface waters.

While this study provides a proof-of-concept, there is an opportunity to improve the process in *P. putida*, perhaps with a further engineered expression system that responds to arsenic in the medium (e.g. (19)). Thus it is this reasonable to propose that methylated arsenicals produced by engineered *pseudomonads* can ameliorate the effects of environmental contamination by inorganic arsenic. In addition, while higher plants do not have an *arsM* gene and do not methylate arsenic, rice transports and accumulates methylated arsenicals produced by paddy microorganisms (20). These organoarsenicals are not only less toxic than inorganic arsenic, but they are much more efficiently translocated from root to shoot and concentrated in the plant tissues. The methylated species can be taken up and concentrated in plants, creating possibilities for rhizo- and phyto-remediation.

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Figure legends

Fig.1. As(III) resistance in *P. putida* expressing *arsM*. Expression of *arsM* confers tolerance to As(III) at the indicated concentrations in M9 medium after overnight growth. Filled symbols: *P. putida* KT2440 pBAM1. Open symbols: *P. putida* KT2440 with integrated *arsM*. Circles, 2 mM As(III); inverted triangles, 7.5 mM As(III); squares, 10 mM As(III). Data are the mean ± SE (n = 3).

Fig.2. Biotransformation of As(III) in *P. putida* expressing *arsM*. A: Cells of *P. putida* KT2440 bearing vector plasmid pBAM1 (control) or *P. putida* KT2440 with *arsM* stably integrated into the chromosome were grown overnight in LB medium with 25 µM As(III). Arsenical species in solution were separated and identified by anion exchange HPLC coupled to ICP-MS. Curve 1, standards. Curve 2: *P. putida* cells bearing pBAM1 incubated with As(III). Curve 3: *P. putida* with integrated *arsM* incubated with As(III). Curve 4: *P. putida* with integrated *arsM* incubated with 25 µM As(V). B: Volatilization of arsenic by *P. putida* with integrated *arsM* for 0 (Curve 1), 12 h (Curve 2) or 48 h (Curve 3) was determined by trapping the gas on H₂O₂-impregnated filters, elution and speciation by HPLC-ICP-MS using an anion-exchange column. C: After 48 h of growth with 25 µM As(III) in LB medium, arsenic species in solution produced by *P. putida* bearing pBAM1 (left) or with integrated *arsM* (right) were quantified by HPLC-ICP-MS. Data are the mean ± SE (n = 3).
References


