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Engineering the Soil Bacterium *Pseudomonas putida* for Arsenic Methylation

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Accumulation of arsenic has potential health risks through consumption of food. Here, we inserted the arsenite [As(III)] *S*-adenosylmethionine methyltransferase (ArsM) gene into the chromosome of *Pseudomonas putida* KT2440. Recombinant bacteria methylate inorganic arsenic into less toxic organoarsenicals. This has the 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 on its Superfund List of Hazardous Substances (http://www.atsdr.cdc.gov/SPL/index.html).

Arsenic is released into the environment by geothermal activity, by dissolution of minerals, and by 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 arsenite [As(III)] *S*-adenosylmethionine (SAM) methyltransferases (termed ArsM in microbes and AS3MT in higher organisms) that biotransform As(III) into methylated species, with volatile nontoxic trimethylarsine [TMAs(III)] (7) as the end product (5, 6, 8, 9). *Pseudomonas putida* is a Gram-negative bacterium found in water and soil, particularly in the rhizosphere at a relatively high population density (10). This microorganism has been studied extensively as a model for biodegradation of aromatic compounds such as naphthalene (11) and styrene (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 the 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 (see Fig. S1A in the supplemental material). After 7 h, methylarsenite [MAs(III)] and dimethylarsenate [DMAs(V)] were produced in relatively equal amounts. After 14 h, the product was primarily DMAs(V), with lesser amounts of trimethylarsine oxide [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_2O_2-impregnated filters by oxidation to TMAs(V)O (see Fig. S1B in the supplemental material). These results demonstrate that purified CrArsM catalyzes three sequential rounds of As(III) methylation and converts toxic inorganic arsenic to less toxic or nontoxic organic arsenicals.

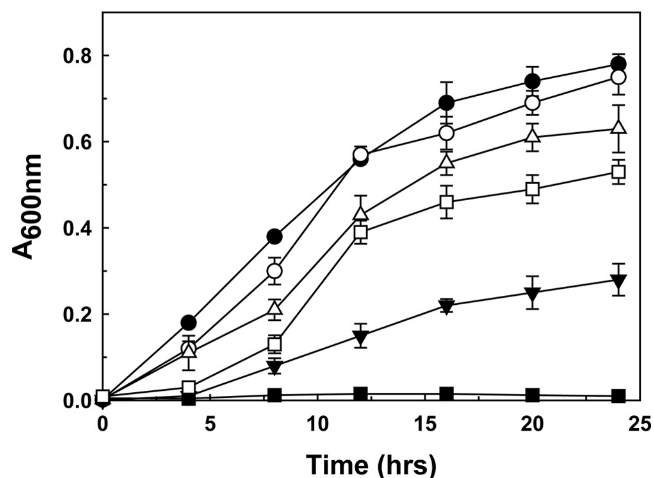

FIG 1 As(III) resistance in *P. putida* expressing *arsM*. Expression of *arsM* confers tolerance to As(III) at the indicated concentrations in M9 medium after overnight growth. Filled symbols, *P. putida* KT2440/pBAM1; open symbols, *P. putida* KT2440 with integrated *arsM*; circles, 2 mM As(III); inverted triangles, 7.5 mM As(III); squares, 10 mM As(III). Data are means ± standard error (SE) ($n = 3$ experiments).

The *C. reinhardtii arsM* gene behind the kanamycin promoter

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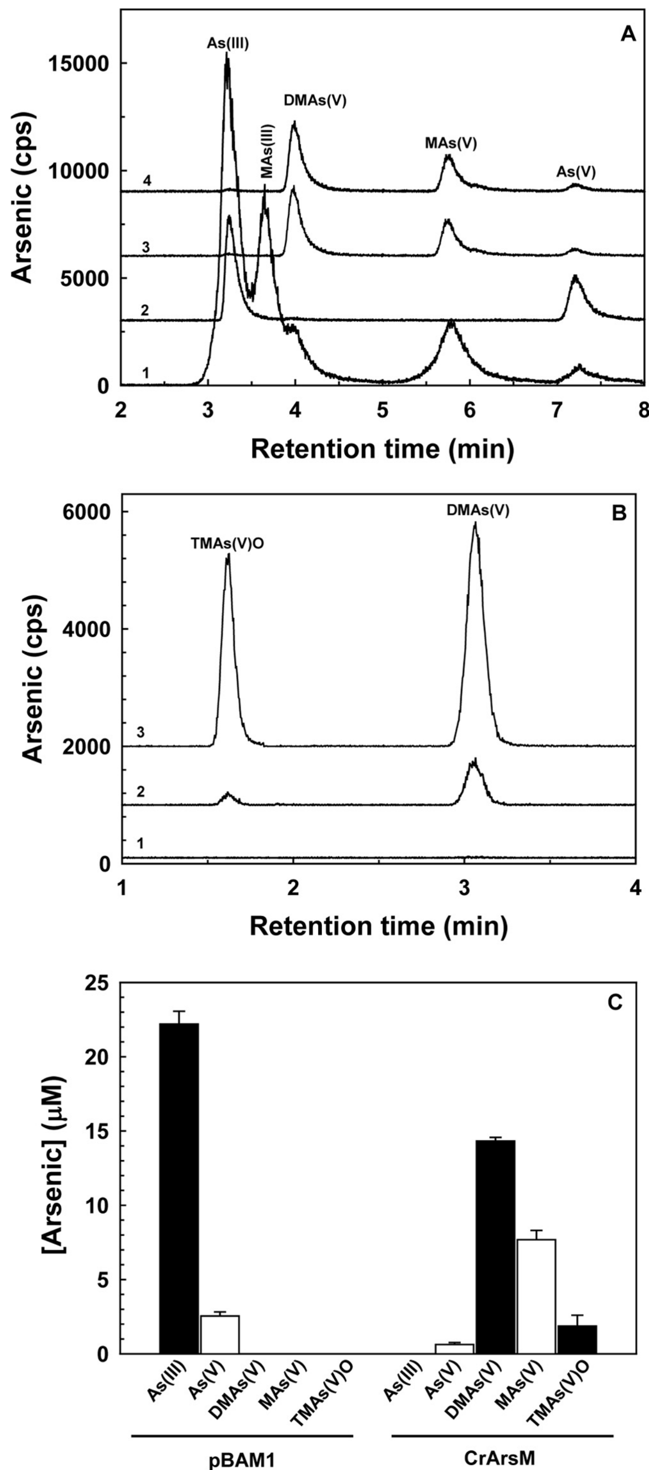

FIG 2 Biotransformation of As(III) in *P. putida* expressing *arsM*. (A) Cells of *P. putida* KT2440 bearing vector plasmid pBAM1 (control) or *P. putida* KT2440 with *arsM* stably integrated into the chromosome were grown overnight in Luria-Bertani medium with 25 μM As(III). Arsenical species in solution were separated and identified by anion-exchange high-performance liquid chromatography (HPLC) coupled to inductively coupled plasma mass spectroscopy (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 H_2O_2-impregnated filters, elution, and separation of species 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 means ± SE ($n = 3$ experiments).

was integrated into the chromosome of *P. putida* KT2440, which does not have an *arsM* gene and does not methylate arsenic. Mini-transposon delivery plasmid pBAM1 was used as a suicide vector to generate stable integrants that could express *arsM* constitutively. The *arsM* gene was cloned into pBAM1 (see Fig. S2 in the supplemental material) and subsequently transferred from *Escherichia coli* CC118λ*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 presence of 2 mM As(III), which provides a competitive advantage to *P. putida* in contaminated soil (10). This could be a 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 salt M9 medium (Fig. 1). Biotransformation of arsenic by the cells was assayed with 25 μM As(III) or arsenate [As(V)] (Fig. 2). After 12 h, engineered *P. putida* biomethylated As(III) primarily to DMAs(V) and, to a lesser degree, methylarsenate [MAs(V)] (Fig. 2A). In a time-dependent fashion, the engineered cells produced dimethylarsine [DMAs(III)H] and TMAs(III) gases, identified by oxidizing them to DMAs(V) and TMAs(V)O with H_2O_2 (Fig. 2B). In addition, the product of the methylation reaction was quantified in cells of *P. putida* expressing CrArsM. After 48 h, the major product found in the culture medium was DMAs(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 DMAs(V) (see Fig. S1A in the supplemental material), while *P. putida* expressing *arsM* produced significant amounts of MAs(V) (Fig. 2A and C). The monomethyl species is the precursor of the dimethyl species (17), indicating that the rate of methylation in *E. coli* is greater than that in *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 (see Fig. 4S in the supplemental material). More enzyme is produced in *E. coli*, most likely because more CrArsM is produced from a plasmid behind the strong T7 promoter than from the chromosomally carried 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 h, the cells transformed nearly all of the inorganic arsenic in the culture medium to the less toxic methylated species, including MAs(V), DMAs(V), 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., see reference 19). Thus, it is 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 phytoremediation.

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