Arsenic-resistant proteobacterium from the phyllosphere of arsenic-hyperaccumulating fern (Pteris vittata L.) reduces arsenate to arsenite

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Abstract: An arsenic-resistant bacterium, AsRB1, was isolated from the fronds of Pteris vittata grown in a site contaminated with copper chromium arsenate. The bacterium exhibited resistance to arsenate, arsenite, and antimony in the culture medium. AsRB1, like Pseudomonas putida, grew on MacConkey and xylose–lactose–desoxycholate agars and utilized citrate but, unlike P. putida, was positive for indole test and negative for oxidase test. A phylogenetic analysis of the 16S rRNA gene showed that AsRB1 is a proteobacterium of the beta subclass, related to Pseudomonas saccharophila and Variovorax paradoxus. Following an exogenous supply of arsenate, most arsenic occurred as arsenite in the medium and the cell extracts, suggesting reduction and extrusion of arsenic as the mechanism for arsenic resistance in AsRB1.

Key words: arsenate reduction, arsenic bioremediation, Pseudomonas saccharophila, Variovorax paradoxus, Pteris vittata.

Arsenic in the environment is a global health problem because of its carcinogenicity. Contamination of soil and water with arsenic could be due to both natural and anthropogenic activities. The Chinese brake fern (Pteris vittata L.) was identified as a hyperaccumulator of arsenic (Ma et al. 2001), leading to novel phytoremediation technologies to remove arsenic from polluted soil (Tu et al. 2002) and water (Huang et al. 2004). Pteris vittata accumulates arsenic in its fronds up to about 6000–7000 mg/kg dry mass (Tu et al. 2002). Studies on the distribution of arsenic within the fronds showed that arsenic was mostly concentrated in the upper and lower epidermal layers (Lombi et al. 2002). We therefore hypothesized that P. vittata phyllosphere could be a potential habitat for arsenic-resistant microorganisms. Because certain microorganisms could enhance the growth and remediation potential of plants (Barac et al. 2004; Burd et al. 1998) and because arsenic-resistant microorganisms could be sources of genes for engineering plants for arsenic resistance and remediation (Dhankher et al. 2002), our objective of the current study was to identify and characterize arsenic-resistant bacteria on the phyllosphere of P. vittata. An arsenic-resistant proteobacterium is described.

Components for bacterial media were purchased from Sigma Chemical Co. (St. Louis, Missouri). Sodium arsenate (Na₂H₂AsO₄·7H₂O) and sodium arsenite (NaAsO₂) were from Mallinckrodt Baker Inc. (Phillipsburg, New Jersey) and antimony potassium tartrate was from Fisher Scientific (Pittsburgh,
Pennsylvania). DNA M<sub>i</sub> marker, Taq polymerase, dNTPs, and pCR 2.1-TOPO cloning kit were from Invitrogen (Carlsbad, California). Oligonucleotide primers were synthesized by the custom primer synthesis unit of IDT DNA Technologies (Coralville, Iowa). Tester strains Staphylococcus epidermidis, Acetobacter aceti, Aeromonas sobria, Enterobacter cloacae, Serratia marcescens, and Pseudomonas putida were purchased from the Carolina Biological Supply Company (Burlington, North Carolina).

Fronds of P. vittata were collected from a site contaminated with 100 ppm of copper chromated arsenate (CCA) in Gainesville, Florida, USA. Fronds were used immediately after harvest. The fronds were placed in 500 mL of 0.9% (m/v) sterile saline solution in a 1000 mL flask and incubated overnight in a shaker set at 160 r/min and 30 °C. The saline solution was then filtered through a 0.2 µm filter, and the cells in the filters were washed into 50 mL of a peptone solution (0.1% m/v). A serial dilution of this suspension was plated on standard plate count agar supplemented with 33 µmol/L As<sub>2</sub>O<sub>5</sub>. Single colonies isolated from these plates were streaked on standard plate count agar with 100 µmol/L As<sub>2</sub>O<sub>5</sub>. After three rounds of single-colony isolations on this medium, the culture obtained on this medium was named AsRB1. Microbiological tests on various media were done as described previously (Collins et al. 1995) with appropriate tester strains. Arsenic resistance was evaluated by growing AsRB1 in liquid Luria–Bertani (LB) medium supplemented with arsenate (As(V)), arsenite (As(III)), or antimony at indicated concentrations and by incubating the cultures at 37 °C for 100 r/min for indicated periods of time. Growth was monitored by turbidimetry at 600 nm in a UV visible spectrophotometer (Beckman, Fullerton, California).

Following exposure of cells to As(V) in the growth medium for specific periods of time, the cells were harvested by centrifugation at 3000g for 10 min. The cell pellet and the medium supernatant were analyzed for total arsenic and for proportions of As(V) and As(III) species using the method suggested by Tu et al. (2004). The bacterial pellet was extracted in 10 mL of methanol–water mixture (1:1, v/v) three times for 2 h each at 60 °C in an ultrasonicator. The three extracts were combined and diluted with water as required. As(V) and As(III) were separated using an As speciation cartridge (Metal Soft Center, Highland Park, New Jersey), which retains As(V). Total arsenic and As(III) were determined by a graphite furnace atomic absorption spectrophotometer (PerkinElmer SIMMA 6000, Norwalk, Connecticut).

Genomic DNA was isolated from AsRB1 using an extraction procedure based on cetyltrimethylammonium bromide (Sambrook et al. 1989). PCR reactions, 50 µL total, contained 10 ng AsRB1 genomic DNA, 20 pmol (each) PCR primer, 40 nmol (each) dNTPs, 125 nmol MgCl<sub>2</sub>, 250 µg bovine serum albumin, and 1 unit Taq polymerase in 1× PCR buffer (Invitrogen). The primers were ribo1492R 5′-GGTTACCTTGTTACGACTT-OH-3′ and ribo27F 5′-AGAGTTTGA TCCTGGCTCAG-OH-3′ (Lane 1991). Following 30 PCR cycles each of 94 °C for 1 min, 50 °C for 45 s, and 72 °C for 2 min, the reaction product was analyzed using an agarose gel electrophoresis. The gel-extracted PCR product was cloned into a pCR 2.1-TOPO vector and sequenced (Sambrook et al. 1989). GenBank accession No. for the clone is AY612302.

The AsRB1 16S rRNA DNA sequence was compared with sequences available in the GenBank database using BLAST (Altschul et al. 1990). Sequence identification and nomenclature of species were based on Anzai et al. (2000). The accession Nos. for 16S rRNA DNA sequences included in the phylogenetic analyses and the corresponding species from which the sequences were derived are as follows: (1) AF233878 Comamonas denitrificans, (2) AJ430348 Comamonas kersteri, (3) AF078766 Acidovorax temperans, (4) AF019073 Hydro-
Fig. 2. Arsenate and antimony resistance of AsRB1 compared with Escherichia coli JM109. Luria–Bertani medium with or without different concentrations of sodium arsenate (A) or antimony potassium tartrate (B) was inoculated with overnight cultures to 0.2 OD_{600} and incubated at 37 °C for 6 h at 200 r/min. Growth measured by turbidimetry at the end of this period was expressed as percent of growth in the control medium without arsenate or antimony.

Multiple sequence alignment was done using ClustalX (Thompson et al. 1997). A phylogenetic tree was drawn using the neighbor-joining method in PAUP (Phylogenetic Analysis Using Parsimony, v4.0b10; Sinaur Associates, Sunderland, Massachusetts). Bootstrap values were obtained by running the PAUP program, and the tree was rooted using midpoint rooting.

When microorganisms washed from P. vittata fronds were plated on standard plate count agar containing As(V), tan-colored bacterial colonies and one fungal species were observed. AsRB1, isolated from the bacterial colonies, was investigated further. This Gram-negative isolate tested positive for indole formation and citrate utilization and grew well on xylose–lactose–desoxycholate agar and MacConkey agar plates. In LB medium, this aerobic bacterium exhibited optimal growth at 37 °C compared with 25, 30, and 42 °C.

Arsenic resistance of AsRB1 was compared with that of a laboratory strain of Escherichia coli. Growth rate of E. coli JM109 at the log phase was reduced 98% by As(V) and...
83.5% by As(III) at a concentration of 833 µmol/L each (Fig. 1). AsRB1’s growth rate was reduced only by 44% and 49% by the same concentration of As(V) and As(III), respectively, (Fig. 1).

When tested with As(V) or antimony in the medium, AsRB1 showed a higher degree of resistance than E. coli JM109 (Fig. 2A). The strains differed in As(V) resistance more than in their antimony resistance (Fig. 2).

The partial gene sequence for 16S rRNA (1484 bp) from AsRB1 was 99% identical to that of Pseudomonas saccharophila, a species related to Leptothrix and Rubrivivax in the Comamonadaceae (Anzai et al. 2000). A phylogenetic analysis of related sequences indicated that AsRB1 belonged to the proteobacterium of the beta subclass (Fig. 3).

In a test to evaluate the uptake and metabolism of As(V) by AsRB1, AsRB1 was cultured in LB liquid medium supplemented with As(V) at 1 mmol/L at 37 °C, and 200 r/min. The cells and the medium were sampled after 6 and 24 h. Total arsenic in the cells was 17 µg/200 mg wet mass and 14 µg/200 mg wet mass, for the 6 and 24 h sampling times, respectively, (Table 1). The wet mass of the cells did not differ significantly between the 6 and 24 h samples. Both the cell extracts and the spent medium contained arsenic. More than 95% of the total arsenic inside the cells and 92% of the total arsenic in the spent medium was in the form of As(III) at both 6 and 24 h sampling times (Table 1).

The fronds of the fern P. vittata provide a unique habitat for arsenic-resistant microorganisms because of the plant’s

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ability to hyperaccumulate arsenic. Although the phyllosphere has been known to be an ideal environment for many microorganisms (Lindow and Brandl 2003), those associated with the arsenic-hyperaccumulating fern have not been characterized. We report here for the first time, the isolation of an arsenic-resistant bacterium from the phyllosphere of P. vittata.

Compared with a laboratory strain of E. coli JM109, AsRB1 exhibited resistance to both As(V) and As(III) (Fig. 1). AsRB1 was also resistant to antimony oxyanions, but it had a better ability to tolerate arsenic than antimony under the assay conditions (Fig. 2). AsRB1’s arsenic resistance could be chromosomal as in Pseudomonas aeruginosa (Cai et al. 1998) or plasmid-borne as in many other microorganisms (Kaur and Rosen 1992). Our attempts to isolate plasmid DNA from AsRB1 using a standard plasmid preparation kit (Qiagen, Valencia, California) failed to yield any DNA (data not shown).

AsRB1 behaved like a proteobacterium in many of the microbiological tests. The phylogenetic analyses of the 16S rRNA gene sequence confirmed that it is a proteobacterium of the beta subclass (Fig. 3).

Many of the bacterial species related to AsRB1 and shown in the phylogenetic tree in Fig. 3 are from varying environments, suggesting that this group of proteobacterium has evolved a wide range of environmental adaptations. For example, Roseatella depolymerans is known to degrade poly(hexamethylene carbonate), Alcaligenes fergusonii to oxidize As(III), Matsuebacter chitosanabidus to produce chitosanase, and Comamonas denitrificans to participate in denitrification (Shimono et al. 1998; Suyama et al. 1998).

When provided with As(V) in the culture medium, AsRB1 cells took up a large percentage of arsenic provided (Table 1). This suggests that AsRB1 has an uptake system for As(V), and it is resistant to arsenic because of its ability to metabolize and (or) extrude arsenic from its cellular compartments.

Because most of the arsenic found in the cell was in the form of As(III), it was inferred that AsRB1 cells reduced As(V) to As(III). This result contrasts to a report of a beta-proteobacterium able to oxidize As(III) to As(V) (Weeger et al. 1999). However, enzymatic reduction of As(V) to As(III) has been characterized in many bacteria, including Staphylococcus aureus (Ji et al. 1994), E. coli (Liu et al. 1995), Pseudomonas aeruginosa (Cai et al. 1998), Bacillus selenitireducens (Oremland et al. 2000), Ferroplasma acidarmanus (Gibirng et al. 2003), and Shewanella sp (Saltikov and Newman 2003). Future studies are required to understand the nature of As(V) reduction in AsRB1.

The finding that a large proportion of arsenic in the medium was As(III) (Table 1) is consistent with the operation of an arsenic extrusion system in AsRB1. Carrier-mediated efflux via both an As(III) carrier protein and As(III)-translocating ATPase are known in bacteria (Rosen 2002). Future studies are needed to understand the nature of As(III) translocation in AsRB1.

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### References


