# Mechanisms of efficient arsenite uptake by arsenic-hyperaccumulator Pteris vittata

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# Bacteria-Mediated Arsenic Oxidation and Reduction in the Growth Media of Arsenic Hyperaccumulator *Pteris vittata*

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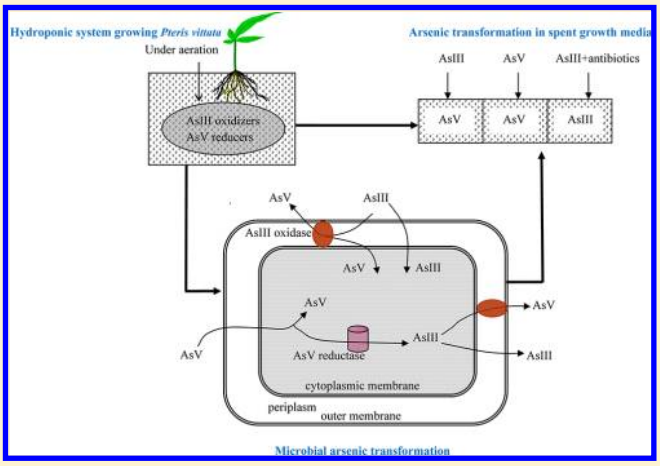
**ABSTRACT:** Microbes play an important role in arsenic transformation and cycling in the environment. Microbial arsenic oxidation and reduction were demonstrated in the growth media of arsenic hyperaccumulator *Pteris vittata* L. All arsenite (AsIII) at 0.1 mM in the media was oxidized after 48 h incubation. Oxidation was largely inhibited by antibiotics, indicating that bacteria played a dominant role. To identify AsIII oxidizing bacteria, degenerate primers were used to amplify ~500 bp of the AsIII oxidase gene *aioA* (*aroA*) using DNA extracted from the media. One *aioA* (*aroA*)-like sequence (MG-1, tentatively identified as *Acinetobacter* sp.) was amplified, exhibiting 82% and 91% identity in terms of gene and deduced protein sequence to those from *Acinetobacter* sp. 33. In addition, four bacterial strains with different arsenic tolerance were isolated and identified as *Comamonas* sp.C-1, *Flavobacterium* sp. C-2, *Staphylococcus* sp. C-3, and *Pseudomonas* sp. C-4 using carbon utilization, fatty acid profiles, and/or sequencing 16s rRNA gene. These isolates exhibited dual capacity for both AsV reduction and AsIII oxidation under ambient conditions. Arsenic-resistant bacteria with strong AsIII oxidizing ability may have potential to improve bioremediation of AsIII-contaminated water using *P. vittata* and/or other biochemical strategies.




## INTRODUCTION

Arsenic, a toxic metalloid, is widely distributed in the environment, which results from both natural and anthropogenic sources.[1] Arsenate (AsV) and arsenite (AsIII) are the two most abundant inorganic arsenic forms in terrestrial and aquatic environments. They are highly toxic to living organisms including microbes, plants, and mammals. Compared to AsV, AsIII is more bioavailable and toxic due to its greater hydrologic mobility and high affinity to reactive sulfhydryl groups of many enzymes involved in crucial cellular metabolism.[2]

Microbial transformations between AsV and AsIII are important in controlling arsenic biogeochemistry and hence arsenic toxicity in various ecosystems.[2] On one hand, AsV can be used as electron acceptor in anaerobic respiration for energy gain.[3] Under aerobic conditions, AsV can be reduced to AsIII followed by extrusion from cytoplasm via specific AsIII transporters, which represents a typical resistance pathway for microbes to cope with high AsV in the environment.[4] On the other hand, AsIII can be oxidized to AsV by both heterotrophic and chemoautotrophic oxidizing bacteria, which have been identified from a wide range of ecosystems and exhibit phylogenetic diversity.[4,5] By transforming AsIII to AsV, AsIII oxidizers attenuate AsIII bioavailability and toxicity in the environment.

Arsenic hyperaccumulator *Pteris vittata* L has a potential to bioremediate arsenic-contaminated soils and water.[6−8] The mechanisms underlying the efficient arsenic uptake, translocation, and cellular detoxification in *P. vittata* have been studied extensively.[9−14] However, microbial transformations of arsenic between AsV and AsIII in the rhizosphere of *P. vittata* have received limited attention. Microbial arsenic transformations play an essential role in controlling arsenic bioavailability and toxicity in the environment and directly affect

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**Table 1. Primer Sequences and PCR Conditions Used in This Study**

| target genes | forward primer | reverse primer | PCR condition | ref |
|---|---|---|---|---|
| *aro*-like gene[a] | 5-GTSGGBTGYGGM TAYCABGYCTA-3 | 5′-TTGTASGCBGG NCGRTTRTGRAT-3′ | 94 °C 2 min, followed by 4 cycles of 95 °C 1 min, 52 °C 45 s, 72 °C 2 min, then 36 cycles of 95 °C 1 min, 50 °C 45 s, 72 °C 2 and 5 min final extension at 72 °C | 18 |
| 16S ribosomal gene | 5′-AGAGTTTGATCM TGGCTCAG-3′ (27F) | 5′-GGTTACCTTGTTA CGACTT-3′(1492R) | 94 °C 2 min, followed by 4 cycles of 95 °C 1 min, 52 °C 45 s, 72 °C 2 min, then another 4 cycles of 95 °C 1 min, 50 °C 45 s, 72 °C 2 min, followed by 32 cycles of 95 °C 1 min, 48 °C 45 s, 72 °C 2 min, and 5 min final extension at 72 °C | 21 |

[a]Standard mixbase codes: $S$ = C/G, $B$ = C/G/T, $Y$ = C/T, $M$ = A/C, $R$ = A/G and $N$ = A/C/G/T.

arsenic uptake and metabolism in plants including *P. vittata*. Furthermore, better understanding of arsenic transformation in *P. vittata* rhizosphere is important to improve bioremediation of arsenic-contaminated soil and water.

Under aerated hydroponic systems, rapid oxidation of AsIII to AsV in the growth media of *P. vittata* has been repeatedly observed.[14,15] For example, 18−67% of the AsIII initially spiked into the growth media of *P. vittata* was oxidized to AsV after 24 h.[15] However, under the same aerated conditions, no AsIII oxidation was observed without *P. vittata*. Furthermore, when growing under aerated sterile conditions, no AsIII oxidation was observed in the growth media of *P. vittata*,[15] indicating the role of microbes in AsIII oxidation. However, AsIII oxidizing microbes were not identified in these studies.

Microbial AsIII oxidation has application in bioremediation by transforming more mobile and toxic AsIII to less toxic AsV, which can be immobilized and removed more easily from contaminated water.[2] Although a wide range of AsIII oxidizers have been reported,[1,16,17] no information is available regarding AsIII oxidizers associated with *P. vittata.*

Our goals were to (1) determine the role of bacteria in AsIII oxidation and AsV reduction in the growth media of *P. vittata* and (2) identify AsIII oxidizers by environmental PCR analysis of metagenomic DNA as well as isolation of specific strains. The results have important implications on the bioremediation of arsenic-contaminated water and soil.

## MATERIALS AND METHODS

Sodium arsenite (Fisher Scientific, Pittsburgh, PA) and sodium arsenate (Sigma Chemical Co., St. Louis, MO) were used to prepare arsenic stock solutions. Microbial media components were purchased from Fisher Scientific. Primers for PCR were custom ordered from Integrated DNA Technologies (Coralville, IA).

**Arsenic Transformation in Growth Media.** Four-month old *P. vittata* plants of uniform size with 4−5 fronds growing in potting media were transferred to 0.2 strength modified Hoagland solution after thorough washing with DI water. They were grown in a controlled environment with an 8 h photoperiod at a light intensity of 350 $\mu$mol $m^{-2}$ $s^{-1}$, 28/23 °C day/night temperature, and 60% relative humidity. Hoagland solution at 0.2 strength without *P. vittata* was kept under the same conditions as control. The growth media with and without *P. vittata* was aerated continuously and changed weekly. After 2 weeks of acclimation, the hydroponic solutions with *P. vittata* growing were separated from the plants and referred to as "spent growth media", which contained bacteria associated with the roots of *P. vittata*.

To investigate arsenic transformation in the spent media, 1 mg $L^{-1}$ AsIII as $NaAsO_2$ or AsV as $Na_2HAsO_4$ was added into the spent media under continuous aeration. Arsenic transformation in the growth media without *P. vittata* (nonsterile) was included as control. Samples were collected periodically. Since only AsV and AsIII are present in the growth media,[15] other arsenic species were not considered in this study. AsV and AsIII in the media were separated using an As-speciation cartridge (Waters Corp., Milford, MA, USA), which retains AsV. Arsenic concentration was analyzed by graphite furnace atomic absorption spectrophotometry (GFAAS; AA240Z, Varian Inc., CA). For As speciation in the growth media of *P. vittata*, analysis using speciation cartridge-GFAAS is comparable to HPLC-ICP-MS.[14,15]

To test the role of microbes in AsIII oxidation, a mixture of antibiotics (100 mg $L^{-1}$ chloramphenicol and 100 mg $L^{-1}$ carbenicillin disodium salt) was added to the spent media. After 72 h preincubation, 1 mg $L^{-1}$ AsIII was added to the media. After 60 h of incubation, arsenic speciation in the media was determined using an As-speciation cartridge and GFAAS.

**AsIII Oxidizing Bacteria Identification via *aioA* (*aroA*)-like Gene Amplification.** Total DNA in the spent growth media (500 mL) was extracted by filtering with a 0.2 $\mu$m filter. A Metagenomic DNA Isolation Kit for aqueous solution (Epicenter Biotechnologies, Madison, WI, USA) was used following the manufacturer's instructions. To amplify *aroA*-like genes, degenerate primers were employed (Table 1), which were designed based on the conserved regions of published AsIII oxidase genes.[18] Genomic DNA from *Agrobacterium* sp. strain LBA4404 was included as a positive control to test the reliability of *aioA* (*aroA)* degenerate primers. The concentration of each primer in 50 $\mu$L PCR system was 1 $\mu$M. PCR products were separated on 0.8% (wt/v) agarose gel, and the target bands were excised, followed by purification (Zymoclean Gel DNA Recovery Kit, Zymo Research, Orange, CA, USA). Purified PCR products were cloned into pCRII-TOPO vector (Invitrogen, Carlsbad, CA, USA). After transformation of competent XL-1 Blue *E. coli* cells and blue/white colony screen, plasmid DNA was extracted from randomly selected transformants using QIAprep Spin Miniprep Kit (QIAGEN Inc. Valencia, CA, USA) followed by sequencing at the Interdisciplinary Center for Biotechnology Research Sequencing Facility at the University of Florida.

**Arsenic Tolerance and Transformation by Bacterial Isolates.** Three media for isolation of culturable bacteria from the spent growth media included the following: (i) *Pseudomonas* basal minimal medium (PBM),[19] with the following composition (1 L, pH ≈ 6.4) 5.00 g of peptone, 0.15 g of ferric ammonium citrate, 0.098 g of $MgSO_4$, 0.066 g of $CaCl_2{\cdot}2H_2O$, 0.05 g of $MnSO_4{\cdot}H_2O$, 0.006 g of $FeCl_3$, and 12 g of agar; (ii) Luria−Bertani (LB) liquid medium; (iii) Hoagland agar medium (1.5% agar), which was prepared from 0.2 $\mu$m filtrated spent growth media with 0.4% (w/v) glucose added as the C source. Spent growth media (200 $\mu$L) was poured onto the three media, and the solution was spread evenly over the surface of each plate, which was then incubated at 30 °C. Isolated colonies were obtained by continuous subculturing and incubation at 30 °C.

To measure their arsenic tolerance, bacterial cultures were inoculated to an initial turbidity of 0.05 $OD_{600\ nm}$ in LB liquid medium (for C1, C3, and C4) or PBM medium (for C2)

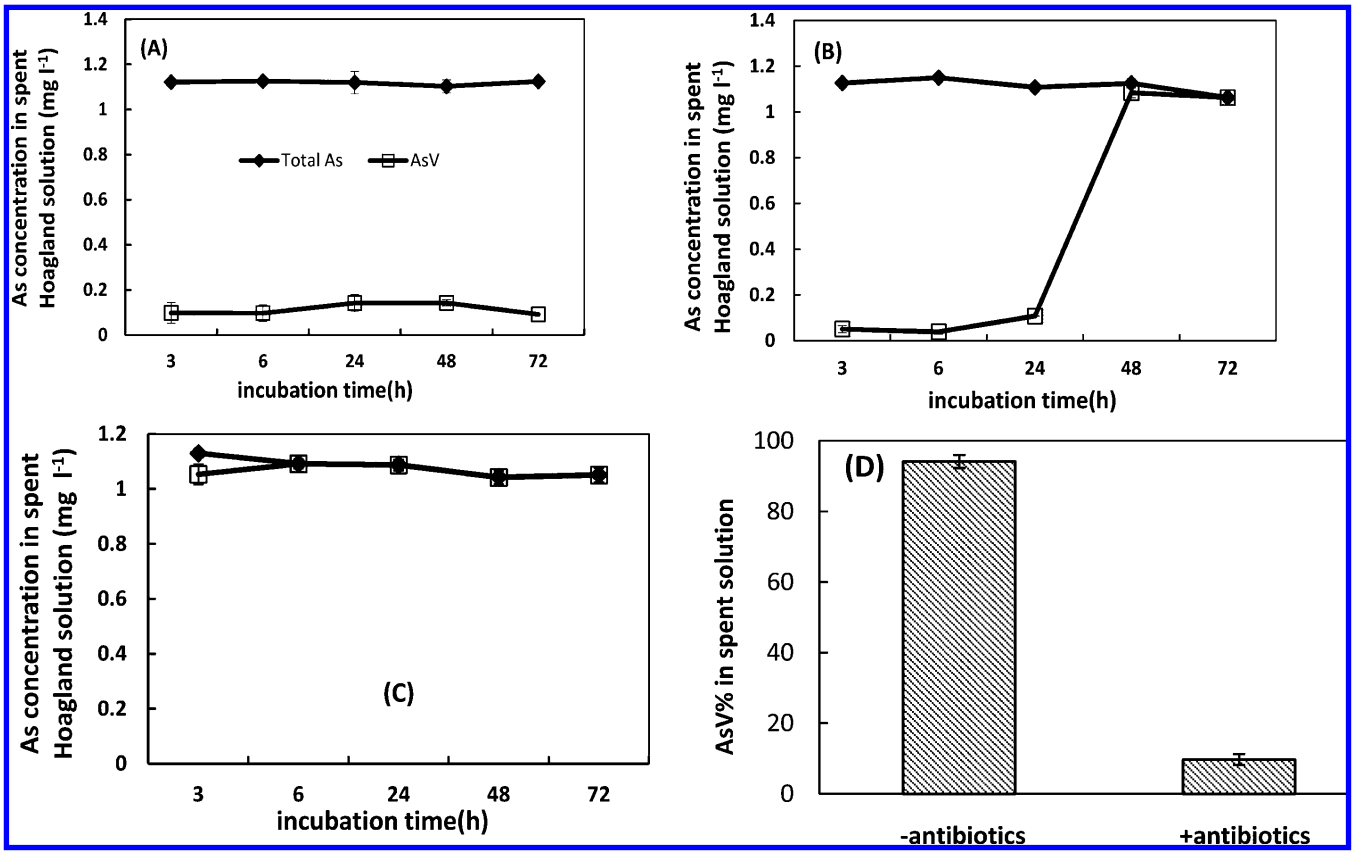


**Figure 1.** Arsenic transformation in (A) fresh 0.2 strength Hoagland growth media, (B) spent 0.2 strength Hoagland growth media spiked with 1 mg $L^{-1}$ AsIII, and (C) spent growth media spiked with 1 mg $L^{-1}$ AsV during 72 h incubation; (D) AsIII oxidation in spent Hoagland growth media spiked with 1 mg $L^{-1}$ AsIII after 60 h incubation with and without antibiotics. Total As = AsV + AsIII. Values are means ± SE ($n$ = 3).

supplemented with 7.5−3000 mg $L^{-1}$ AsV or 3.75−75 mg $L^{-1}$ AsIII. Bacterial growth was measured following incubation at 30 °C, 200 rpm for 12 h.

To test AsIII-oxidizing and AsV-reducing abilities of the isolated strains, pure bacterial cultures at an early log phase growth were inoculated into LB liquid medium (for C1, C3, and C4) or PBM medium (for C2) containing 7.5 mg $L^{-1}$ AsIII or AsV. Control treatment was included by adding 7.5 mg $L^{-1}$ AsIII or AsV to the sterilized media. After 4 h of growth at 30 °C with 200 rpm, $OD_{600\ nm}$ of the cultures was measured. Bacterial cells were collected by centrifugation at 14 000$g$ for 2 min. Arsenic speciation was carried out in the supernatant fluids of different cultures and the corresponding extracts of cell pellets after breaking the cells via ultrasonication and extraction into 50% methanol using an As-speciation cartridge and GFAAS as described before.

**Identification of Bacterial Isolates Using Biolog Metabolic Fingerprinting and Fatty Acid Methylester Analysis.** Suspensions of bacterial isolates in sterile saline solution were used to inoculate Biolog GN (Biolog Inc., Hayward, CA) and GP microplates (150 $\mu$L per well). The plates were incubated for 24 h at 28 °C and read with a spectrophotometer at 590 nm. Reactions in the wells were estimated using the data-dependent threshold algorithm using the MicroLog 4.2 software.

Fatty acids were extracted in xylene and saponified in dilute sodium hydroxide/methanol solution followed by treatment with dilute HCl/methanol solution to derive respective methyl esters (FAMEs) using MIDI Sherlock (Midi Inc., Newark, DE). The FAMES extracted from the aqueous phase were analyzed by a gas chromatograph and interpreted using MIDI Sherlock software.[20]

**16S rRNA Gene Sequencing of C1 Bacterial Isolate and Phylogenetic Analysis.** Bacterial genomic DNA was extracted from the cells collected by centrifugation of overnight C1 cultures using an isolation kit for metagenomic DNA from water (EPICENTRE Biotechnologies). 16s rRNA gene sequences were PCR amplified with Taq polymerase and 27F and 1492R primers[21] (Table 1). Degenerate primers targeting *aioA*-like sequences (Table 1) were also used to identify AsIII oxidase genes of C1 with varying $Mg^{2+}$ concentrations (1, 2, and 4 mM) and annealing temperatures (48−54 °C). PCR products were cloned using a TOPO TA Cloning Kit (Invitrogen Inc.) and sequenced at the Interdisciplinary Center for Biotechnology Research Sequencing Facility at the University of Florida. The phylogenetic relationship between C1 and the reference strains was implemented in MEGA 4.1.

The aioA (aroA) sequence from MG-1 has been deposited in the GenBank database under accession no. HQ188910, and the 16S rRNA gene sequences from *Comamonas sp.*C-1 and *Flavobacterium sp.*C-2 have been deposited under accession nos. HQ188911 and HQ188912, respectively.

**Data Analysis.** All values were expressed as means ± SE ($n$ = 3). Ttreatment effects were determined by analysis of variance according to the linear model procedure of the Statistical Analysis System (SAS Institute Inc., 1986). Treatment means were separated by Duncan's multiple range tests using a level of significance of $p < 0.05$. Protein sequence alignment and phylogenetic analysis were implemented in MEGA 4.1.

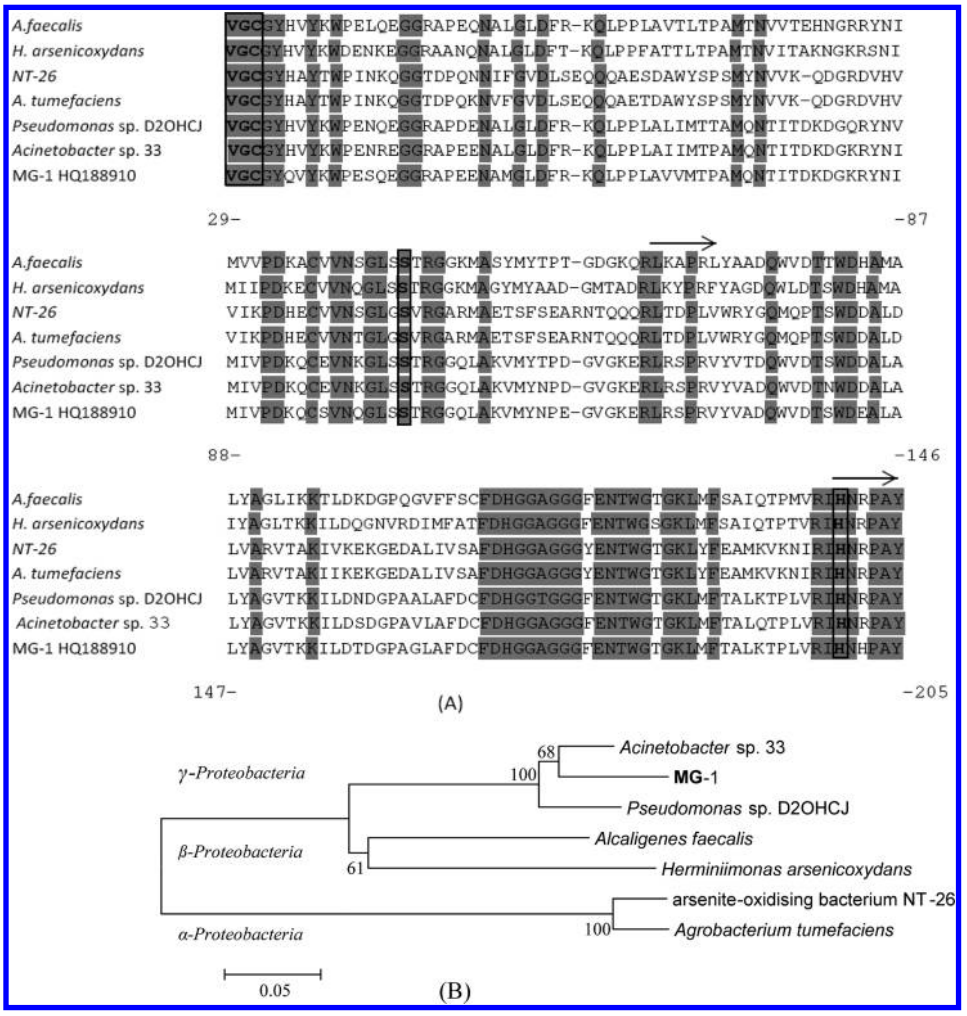


**Figure 2.** (A) Protein sequence alignment of AsIII oxidase deduced from *aroA* genes. Sequence alignment was carried out by MEGA 4.1. Amino acid residue numbers are defined from the first residue (valine) encoded by *aroA* genes amplified from MG-1 in the present study, corresponding to No. 29 in AroA protein sequence in *A. tumefaciens*. Conserved residues among the five protein sequences are in gray. Residues known to play a role inAsIII oxidase activity are framed in bold black. Black arrows at positions 125 and 201 indicate the beginning of domains II and III, respectively. (B) Neighbor-joining phylogenetic tree based on AroA protein sequences showing the phylogenic relationship between MG-1 and the known AsIII oxidizers. Scale bars correspond to 5 mutations per 100 residues. Phylogenetic programs were implemented in MEGA 4.1. GenBank accession no. *Alcaligenes faecalis* AY297781, *Herminiimonas arsenicoxydans* AF509588, *Agrobacterium tumefaciens* DQ151549, arsenite-oxidizing bacterium NT-26 AY345225, *Pseudomonas* sp.D2OHCJ EU304276.1, *Acinetobacter* sp. 33 EU304275.1, MG-1 HQ188910.

## RESULTS AND DISCUSSION

**Arsenic Oxidation and Reduction in the Spent Growth Media.** With low levels of easily degradable organic carbon from the root exudates of *P .vittata*, the growth media provided natural conditions favoring bacteria growth, resulting in ocherous color in the media after 1 week of growth (data not shown). Rapid AsIII oxidation has been observed in the growth media of *P. vittata*,[14,15] which was confirmed in the present study.

After 72 h incubation of 1 mg $L^{-1}$AsIII under aeration, little AsIII oxidation was observed in the control media without *P. vittata* (Figure 1A). In contrast, complete oxidation of AsIII occurred in the spent media where *P. vittata* grew for 2 weeks before collection (Figure 1B). Unlike AsIII, AsV in the spent media was stable with no reduction (Figure 1C).

To test the possible role of bacteria in AsIII oxidation in the spent media, a mixture of antibiotics comprising chloramphenicol and carbenicillin was used. Addition of antibiotics significantly decreased AsIII oxidation rate, with only 10% of the initial AsIII (1 mg $L^{-1}$) being oxidized after 60 h incubation compared to 90% in the media without antibiotics (Figure 1D). The results suggest that bacteria were mostly responsible for AsIII oxidation in the spent media. Since the bacteria in the spent media were originally from *P. vittata* roots and little AsIII oxidation occurred in the control solution without *P. vittata*, it is conceivable that specific rhizosphere bacteria played a dominant role in AsIII oxidation. Recently, a diverse range of AsV reducers has been isolated from the rhizosphere of *P. vittata* growing in arsenic-contaminated sites;[22,23] however, rhizosphere bacteria capable of AsIII oxidation under aerated hydroponic system have not been explored before.

**Identification of AsIII Oxidizers Using Total Environmental DNA.** By using degenerate primers designed based on the conserved region encoding the Mo-pterin subunit of AsIII oxidase from AsIII oxidizing bacteria available in GenBank (Table 1), one *aioA*(*aroA*)-like sequence was successfully obtained from the spent media, which exhibited a high sequence identity value of 82% for nucleotide (and 91% for the deduced protein) to AsIII oxidase large subunit in *Acinetobacter* sp. 33 (EU304275.1). Therefore, the corresponding bacterial species containing *aioA*-like gene was named MG-1.

**Table 2. Identification of Bacterial Isolates Based on Metabolic Fingerprinting Using Biolog System and Fatty Acid Methylester Analyses (FAME)[a]**

| | metabolic fingerprinting using Biolog system | | | | | fatty acid methylester analyses | |
|---|---|---|---|---|---|---|---|
| bacterial isolate | identification | plate type | prob. | dist. | SIM | identification | SIM |
| C1 | *Comamonas testosteroni* | GN2 | 100 | 1.56 | 0.895 | *Comamonas testosteroni* | 0.554 |
| C2 | *Flavimonas oryzihabitans* | GN2 | 99 | 0.62 | 0.944 | *Flavimonas oryzihabitans* | 0.877 |
| C3 | *Staphylococcus epidermidis* | GP2 | 96 | 1.47 | 0.868 | *Staphylococcus capitis/Staphylococcus epidermidis* | 0.659/0.651 |
| C4 | *Pseudomonas alcaligenes* | GN2 | 100 | 7.17 | 0.545 | *Pseudomonas alcaligenes* | 0.684 |

[a]In Biolog system, species identification, plate type (Gram negative GN2 and Gram positive GP2), probability (Prob), distance (DIST), and SIM values are from the Microlog 4.2 output. In fatty acid methyl ester analysis, similarity index (SIM) calculated by the MIDI Sherlock microbial identification system is given with two values being presented if SIMs for the top two matches did not differ by 0.1.

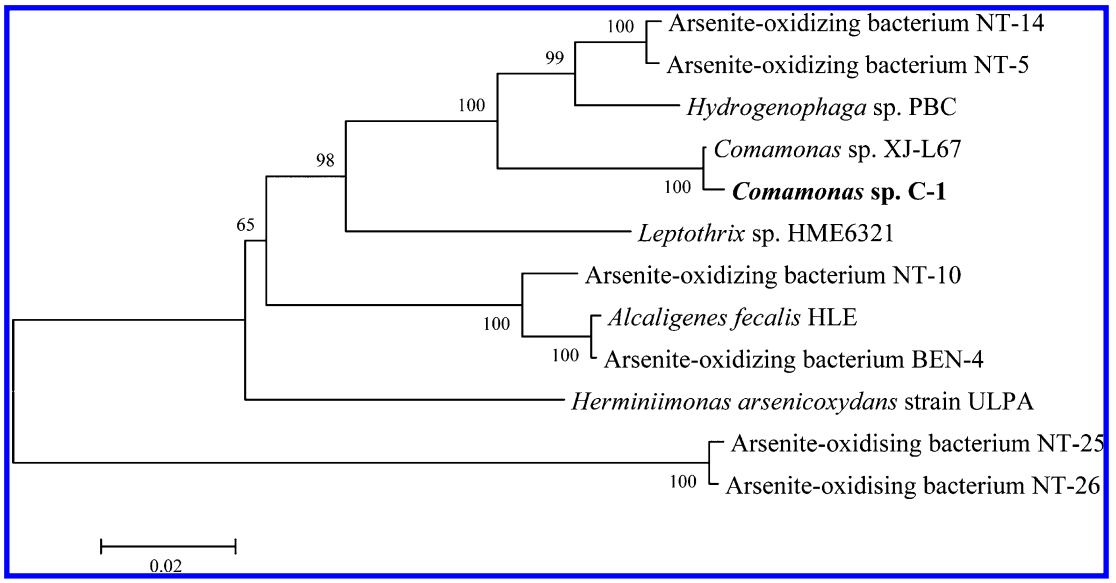


**Figure 3.** Neighbor-joining phylogenetic tree based on partial 16S ribosomal genes showing the positions of *Comamonas* sp.C-1 (HQ188911) identified in the present study compared with species belonging to $\beta$-Proteobacteria. Scale bars correspond to 2 mutations per 100 nucleotides of 16S rRNA sequence. Phylogenetic programs were implemented in MEGA 4.1. GenBank accession no. *Alcaligenes faecalis* HLE AY027506, *Alcaligenes faecalis* NT-25 AF159452, *Alcaligenes faecalis* NT-26 AF159453, AsIII oxidizing bacterium NT-5 AY027498, AsIII oxidizing bacterium NT-10 AY027500, AsIII oxidizing bacterium NT-14 AY027497, AsIII oxidizing bacterium BEN-4 AY027504, *Herminiimonas arsenicoxydans* strain ULPAs1 AY728038.1, *Hydrogenophaga* sp. PBC HM194607.1, *Leptothrix* sp. HME6321 HM590834.1, *Comamonas* sp. XJ-L67 EU817492.1.

Analysis of the deduced protein sequence showed that the AioA (AroA) of MG-1 had a high degree of amino acid conservation as compared with the known AioA (AroA) protein sequences including the residues known to play critical roles in AsIII oxidase activity (Figure 2A). The motif C-$X_2$-C-$X_3$-C-$X_{70}$-S, by which the [3Fe-4S] cluster is coordinated,[24] was conserved between MG-1 and four known AioA sequences (Figure 2A), particularly between residuals 29 and 38 and between residuals 91 and 110. Two conserved domains (II and III) in *A. faecalis* protein, which contain several structural motifs,[25] were also present unchanged in the AioA subunit of MG-1 (residues 124–205) (Figure 2A). Although the HNRPAYNSE motif has been shown to be highly conserved in most bacterial AsIII oxidases,[24] substitution of R with H (residue 202) was observed in AioA subunit of MG-1 (Figure 2A), while residues S103 and A204 in the AioA subunit of MG-1, corresponding to S99 and A199 in *A. faecalis*, were exactly conserved (Figure 2A), which are critical to differentiate the structure of AsIII oxidase from other Mo enzymes of the DMSO reductase family.[25] A phylogentic analysis of the amino acid sequences indicated that MG-1 belongs to the *Pseudomonadales* in Gammaproteobacteria and formed a sister clade to *Pseudomonas* sp. D2OHCJ (Figure 2B).

PCR amplification of *aroA*-like genes using metagenomic DNA extracted directly from the spent media provided a culture-independent approach to uncover a natural diversity of *aioA*-like genes in the concerned environmental system, which may not be accessible and detectable by traditional culture methods. However, this method was of limited utility because despite the use of degenerate primers a wide array of AsIII oxidizers was not identified.

**Arsenic Tolerance and Transformation by Bacterial Isolates.** Following plating of the spent media, after 2 days of incubation at 30 °C, four colonies with different morphologies appeared on the PBM medium and LB medium. Purified cultures were obtained by continuous substreak and were named C-1, C-2, C-3, and C-4. Among the four strains, C-2 displayed a yellow color with others being white or tan, and none exhibited fluorescence under UV. Three of the four bacterial isolates grew well in LB medium, but C-2 grew best in PBM medium.

Carbon utilization assay using Biolog plates showed that C1, C2, and C3 had high similarity indices to *Comamonas testosterone*, *Flavimonas oryzihabitans*, and *Staphylococcus epidermidis*, respectively, and C4 had a relatively lower similarity index to *Pseudomonas alcaligenes* (Table 2). Fatty acid methyl ester analyses were consistent with these identifications, matching C1

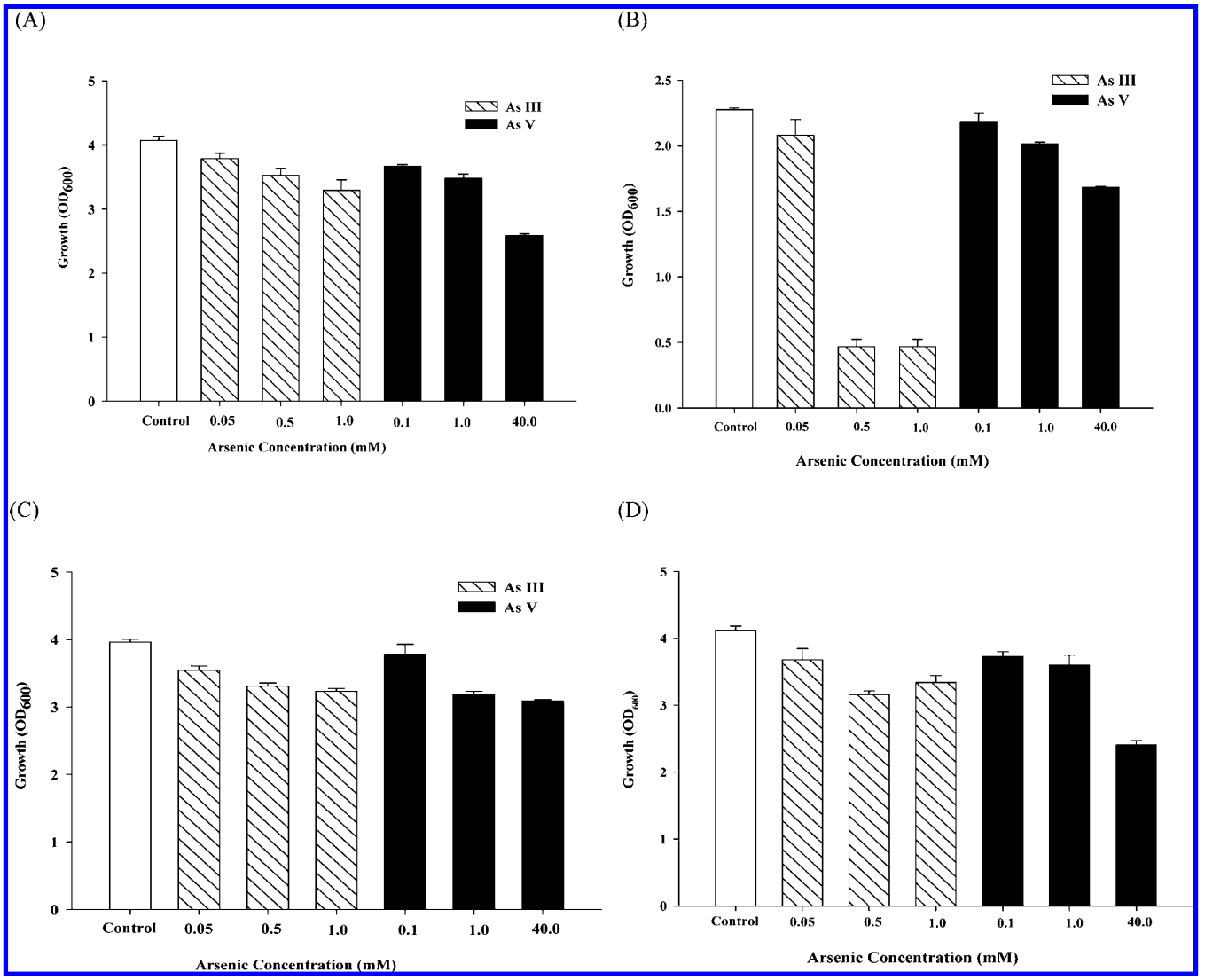


**Figure 4.** Arsenic resistance of bacterial isolates C1, C2, C3, and C4. Late log phase cultures of C1 (A), C2 (B), C3 (C), and C4 (D) were inoculated to 0.05 $OD_{600}$ density in LB medium (C1, C3, and C4) or Pseudomonas Basal Mineral medium (C2) containing 7.5−3000 mg $L^{-1}$ (0.1−40.0 mM) AsV (solid bars) or 3.75−75 mg $L^{-1}$ (0.05−1.0 mM) AsIII (striped bars) or no arsenic control (open bars) and incubated for 12 h at 200 rpm at 30 °C. Optical density at 600 nm was measured using a spectrophotometer. Values are means ± SE ($n$ = 3).

to *Comamonas testosterone* and C2 to *Flavimonas orhzihabitans* (Table 2). C3 matched with both *Staphylococcus capitis* and *S. epidermidis* with close similarity indices and C4 with *P. alcaligenes* (Table 2). In addition, according to 16s rRNA gene sequence analysis, C-1 exhibited 99% identity to that of *Comamonas* sp. XJ-L67 (Figure 3).

All four isolates were resistant to 3.75 mg $L^{-1}$ AsIII with ∼10% growth reduction compared to the control (Figure 4). When tested at 75 mg $L^{-1}$ AsIII, C-2 was significantly more sensitive than C-1, C-3, and C-4 (Figure 4). All four isolates had high tolerance to AsV based on their growth in the media containing 3000 mg $L^{-1}$ AsV (Figure 4). While C-1, C-3, and C-4 were equally tolerant to 75 mg $L^{-1}$ AsIII or AsV, C-2 was ∼4 times more tolerant to AsV than AsIII (Figure 4B).

To test their ability in AsIII oxidation, the four strains were inoculated into flasks with liquid media (LB media for C1, C3, and C4 and PBM medium for C2) containing 7.5 mg $L^{-1}$ AsIII. After 4 h of incubation under 200 rpm, compared to the control (∼10%), significantly more AsIII (∼27%) was oxidized in the media where C-1 was inoculated but C-2, C-3, and C-4 showed little difference (Figure 5A). AsIII oxidation in the control media (sterilized LB or PBM) could be caused by the strong aeration under 200 rpm shaking conditions. To further test AsIII oxidizing capacity of the four isolates, arsenic speciation in the cell pellets was analyzed following 4 h incubation with 7.5 mg $L^{-1}$ AsIII. Of the total arsenic, 34%, 55%, 24%, and 70% was AsV in C-1, C-2, C-3, and C-4, respectively (Figure 5B). Together these results suggest that the four isolates had differing abilities to oxidize AsIII with C1 being the most efficient.

When tested for AsV reduction, no detectable AsIII was observed in the control media. However, a small but detectable fraction AsIII (5.6%, 0.5%, 2.6%, and 1.6%) appeared in the spent media inoculated with C-1, C-2, C-3, and C-4, respectively (Figure 6A). Analysis of the cell pellet extracts showed that C-1 cells contained significantly greater AsIII levels (17% of the total arsenic) than C-2 (4.2%), C-3 (4.5%), and C-4 (13%) (Figure 6B). Together these results showed that the four isolates had differing abilities to reduce AsV as well again with C1 being the most efficient.


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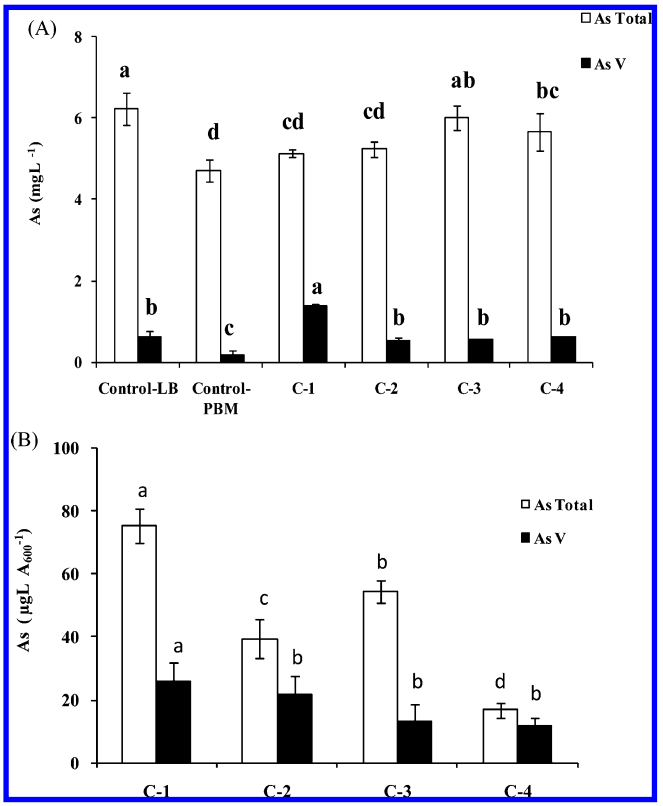


**Figure 5.** AsIII oxidation by bacterial isolates C1, C3, and C4 in LB growth media and C2 in PBM growth media containing 7.5 mg $L^{-1}$ (0.1 mM) AsIII during 4 h incubation. Total arsenic (open bars) and arsenate (AsV; solid bars) were determined in the spent growth media (A), and corresponding extracts of bacteria cells are expressed per unit cell density (B). Total As = AsV + AsIII. Values are means ± SE ($n$ = 3). Bars denoted with different letter codes are significantly different at $p$ = 0.05.

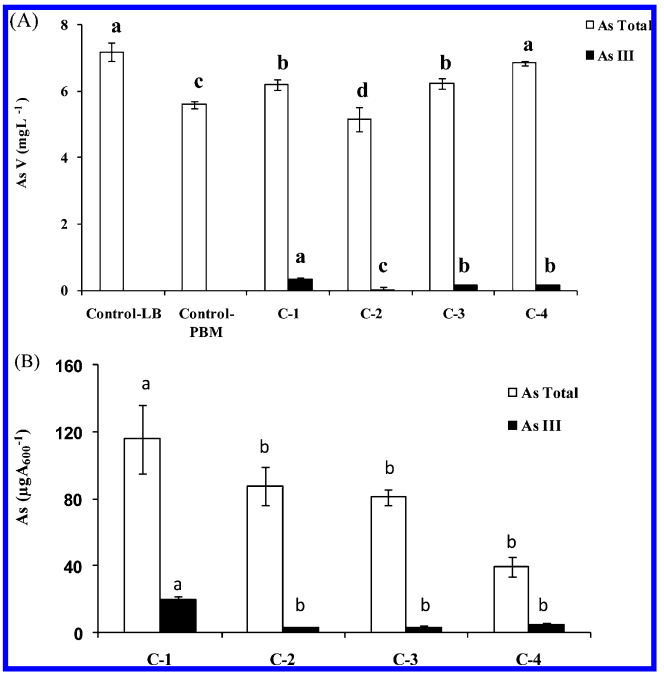


**Figure 6.** AsV reduction by bacterial isolates C1, C3, and C4 in LB growth media and C2 in PBM growth media containing 7.5 mg $L^{-1}$ (0.1 mM) AsV during 4 h incubation. Total arsenic (open bars) and AsIII (solid bars) were determined in the spent growth media (A) and corresponding extracts of bacterial cells, expressed per unit cell density (B). Total As = AsV + AsIII. Values are means ± SE ($n$ = 3). Bars denoted with different letter codes are significantly different at $p$ = 0.05.

In addition, when we tried to amplify *aioA*-like genes from *Comamonas* sp. C-1 with varying $Mg^{2+}$ concentrations (1, 2, and 4 mM) and annealing temperatures (48−54 °C) in PCR system, no *aioA*-like genes could be amplified. This could have been merely due to the primers employed were not quite suitable for this species. However, an alternative explanation is that AsIII oxidase in this species had divergent sequence or AsIII oxidation occurred by a novel mechanism. This is similar to the study of Hoeft et al.,[26] who obtained no authentic PCR product for AsIII oxidase from the extracted DNA of a hot spring biofilm bacterial community in spite of its remarkable AsIII oxidation activity being observed. Specifically, the high similarity index of *Comamonas* sp. C-1 to *C. testosterone* (Table 2), which contains AsV reductase gene in its genome,[27] indicates the former may function as an AsV reducer under specific conditions. This was confirmed by the present experiment where *Comamonas* sp. C-1 induced not only significant oxidation of AsIII at 7.5 mg $L^{-1}$ after 4 h of incubation (Figure 5) but also substantial reduction of AsV at 7.5 mg $L^{-1}$ to AsIII when provided with AsV (Figure 6).

To further investigate whether AsIII oxidation by C-1 was by the cells or by enzymatic and nonenzymatic factors released from the cells, we tested AsIII oxidation in the spent media where C-1 cells were cultured initially. AsIII oxidation in such a spent medium was no more than that of the background control (data not shown), suggesting that AsIII oxidation by C-1 was actively mediated by bacterial cells. Consistent with our results we propose that C-1 bacteria tolerated high levels of AsV by reducing AsV within the cells and exporting AsIII out. We hypothesize that an AsIII-oxidase, spatially separated from AsV-reductase, oxidized the AsIII while it was exported outside the cells. This is consistent with our observation that only a small fraction, i.e., 5.6%, of total arsenic was present as AsIII in the media spiked with AsV (Figure 6A).

In this study despite our identification of bacteria related to *Acinetobacter* sp. by analyzing the metagenomic DNA culture methods identified other taxa only. This could be due to low abundance of *Acinetobacter* sp. and/or nonoptimal isolation conditions for this species.

**Environmental Implications.** Microbial AsIII oxidation capacity in the rhizosphere of *P. vittata* can be explored to preoxidize AsIII in contaminated water in a cost-effective manner followed by efficient removal of AsV via plant hyperaccmulation or physiochemical precipitation/adsorption. Therefore, the symbiosis of rhizosphere AsIII oxidizers and *P. vittata* roots has potential advantages as an effective bioaugmentation technology[28] with the plant roots supporting bacterial growth and increasing the likelihood for their survival. Comparatively, AsV uptake rate by *P. vittata* was up to 9−20 times higher than that of AsIII in the absence of P.[9] This suggests that with low P content, AsIII removal efficiency could be increased by synergic function of microbial AsIII oxidation in the rhizosphere followed by more rapid root uptake of AsV. In addition, some *Commamonas* isolates previously identified exhibited potential for accelerated degradation of diverse organic contaminants.[29,30] Whether the rhizosphere system of *P. vittata* can play a role in degrading toxic organic pollutants in As-contaminated water is raised as a new issue deserving further study.

In conclusion, AsIII oxidizers identified from the growth media of *P. vittata* in a hydroponic system exhibited versatile resistance to both AsIII and AsV, which greatly facilitated the survival of


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these rhizobacteria when exposed to different arsenic species under varying ambient conditions. Particularly, *Comamonas* sp. C-1, with remarkable capacity in both AsIII oxidation and AsV reduction, could provide insight into novel mechanisms of arsenic detoxification in microorganisms. In the future, AsIII oxidizers from *P. vittata* roots could be applied to improve the bioremediation efficiency of AsIII-contaminated water and decrease AsIII bioavailability and toxicity in various contaminated ecosystems.

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### Notes

The authors declare no competing financial interest.

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