INFLUENCE OF ARSENIC RESISTANT BACTERIA ON SOLUBILIZATION OF ARSENIC AND PHOSPHORUS MINERALS AND THEIR UPTAKE BY PLANTS

By

PIYASA GHOSH

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This dissertation is dedicated to my mother, Anupama Ghosh for fostering in me a love for acquiring knowledge and the determination to achieve it.
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By

Piyasa Ghosh

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Cochair: Bala Rathinasabapathi
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Arsenic contamination in soil and groundwater has created serious health concern around the world. Arsenic-hyperaccumulator *Pteris vittata* L. can accumulate up to 2.3% arsenic (As) in its biomass, making it a viable candidate for phytoremediation of As-contaminated sites. Seven arsenic-resistant siderophore-producing bacteria (ARB) belonging to genera *Pseudomonas* sp., *Comamonas* sp. and *Stenotrophomonas* sp. tolerant to 10 mM As(V) were isolated from the rhizosphere of *P. vittata*. We tried to understand the beneficial relationship between the bacteria and *P. vittata* and utilize this knowledge to improve growth in other plants like tomato.

We tested P uptake and As(V) reduction rates at 0.1 and 1 mM As(V) in ARB. ~80-88% P uptake took place in first 4 h of incubation when less than 9% As(V) was reduced that increased to 47-90% in 12 h. ~3.0-8.4 times more bacterial P uptake occurred at 1 mM than at 0.1 mM As(V) due to As-induced P uptake. Aerobic ARB were capable of both As(III) oxidation and As(V) reduction. Higher siderophore-producing bacteria were more efficient in As(III) oxidation (r=0.77) and less efficient in As(V) reduction (r= -0.81). The nonsiderophore-producing mutants of *Pseudomonas*
*fluorescens* Pf-5 were 23-25% less tolerant to As(V) and As(III) compared to the wild-type.

Solubilization of As from FeAsO$_4$/AlAsO$_4$ minerals by ARB siderophores, increased As uptake by *P. vittata* from 18.1–21.9 to 35.3–236 mg kg$^{-1}$ As in fronds and the root biomass of from 1.5-2.2 to 3.4-4.2 g dw/plant. This increase in biomass by ARB was associated with arsenic-induced P uptake. PG6 solubilized most amount of P (0.8 mg L$^{-1}$) from phytate and PG12 (9.05 mg L$^{-1}$) from FePO$_4$ after 1 d of incubation. Addition of spent medium from PG6 or PG12 to 0.2X Hoagland’s medium of tomato supplied with phytate increased its shoot biomass by 1.7 times (0.04 g dw/plant) and in FePO$_4$ treatment by 2.3 times (0.22 g dw/plant) after 7 d of growth. Our results suggest that the bacterial isolates can be used in improving As phytoextraction by *P. vittata* and their exudates containing siderophores can enhance mineral uptake by other crop plants.
CHAPTER 1
INTRODUCTION

The goal of my research was to improve the efficiency of arsenic (As) uptake by the As hyperaccumulating fern *Pteris vittata* L and iron (Fe) and phosphate (P) uptake by the crop plant tomato. For this purpose I studied the interaction between arsenic resistant rhizobacteria associated with the roots of *P. vittata* and the insoluble Fe minerals that also affected arsenic tolerance in bacteria. We investigated the role of siderophores in releasing phosphorus from insoluble Fe-minerals to improve iron and phosphorus nutrition in food plants like tomato.

In the first chapter, literature review is provided that helped me formulate research objectives. The following four chapters deal with the experimental data that I generated to answer the research questions. The second chapter deals with the details of study site locations and determination of soil properties, isolation of bacteria and characterization of siderophores. The next chapter deals with effect of arsenic oxidation and reduction by As resistant bacteria. The next two chapters describe the effect of bacteria on solubilization of soil minerals and their effect on plant growth in *Pteris vittata* and *Solanum lycopersicum* (tomato). The last chapter discusses our major experimental accomplishments and the scopes for future research.

**Arsenic: Source, Speciation and Bioavailability**

Arsenic pollution causes serious health problems in millions of people around the world. In natural soils, As is commonly concentrated in sulfide-bearing mineral deposits, like gold mine deposits and ores associated with pyrite, one of the ubiquitous minerals in the Earth’s crust (Nriagu 2002). Arsenic is abundant in hydrous iron oxides, and the
toxic element can easily be solubilized in ground waters depending on pH, redox conditions, temperature, and solution composition (Islam et al. 2004).

Arsenic compounds can exist as inorganic and organic form. Arsenic exists in the following valence states: As(0), arsenite As(III), arsenate As(V) and arsine gas (-3) (Oremland and Stolz 2003). Human exposure to As may take place via food, water, soil, and air. The extent of As toxicity is controlled largely by the speciation and solubility of the inorganic species. In the environment, As may be released in the ground water by weathering of mineral ores and other geothermal processes (Smedley and Kinniburgh 2002). In air, As may be added by volcanic eruption (Signorelli 1999). Human interaction can also cause As pollution through industrial processes involving smelting of metal ores (Smedley and Kinniburgh 2002). For example, arsenic trioxide was used in pesticides and defoliants. Pressure-treated wood also contained high amount of As. Presently, gallium arsenide and arsine gas are still used in the electronics industry as components of semiconductor devices. In the medical industry, As is also used in chemotherapy for treatment of acute leukemia. Organic As may also be found in certain food like seafood (http://www.atsdr.cdc.gov/).

Arsenic intake from drinking water is of major health concern across the world as it can cause skin and lung cancers and other diseases like diabetes, skin diseases, nervous and cardiovascular problems (Vahter 2007). Arsenic, present as As(III) and As(V), is soluble in water and hence bioavailable for uptake by plants and animals. As(V) after entering into the cell may replace phosphorus (P) in biochemical pathways as it is a structural analog of P and inhibit oxidative phosphorylation, which is life’s main energy producing system. On the other hand, As(III) is considered to be more broadly
toxic as it can bind to the sulfhydryl groups in the proteins and be retained as organic As (Oremland and Stolz 1999). Arsenite can also affect respiration by binding to the thiol groups in the respiratory enzymes like pyruvate dehydrogenase and glutarate dehydrogenase (National Research Council 1999). Recently, As(III) has also been reported to interact with glucocorticoid receptor (Kaltreider et al. 2001, Oremland and Stolz 2003). The As standard in drinking water by WHO is 10 µg L⁻¹. This requires employment of efficient and cost-effective remediation techniques for clean-up of As-contaminated soil and water.

**Pteris vittata: Arsenic hyperaccumulator**

The existing conventional methods of remediating As-contaminated soil and water include oxidation, coagulation, ion exchange, reverse osmosis and electrodialysis. Phytoremediation is an emerging, green technology, which is more cost effective and applicable for clean-up of As-contaminated soils (Leung et al. 2006) and water (Natarajan et al. 2008). Plants show different tolerance to metal stress. Some may be non-accumulators, others may be unable to tolerate the influx of metals, and still others may be hyperaccumulators (Xie et al. 2009). Some plant species have an unusual capacity to tolerate and even thrive in metal-contaminated soils (Zhao et al. 2009). Few of these plants accumulate high levels of the metal in their tissues, earning them the title ‘hyperaccumulator’. These hyperaccumulating plants can be used to remove metals from soil, and transfer it to their biomass. The following three properties distinguish a successful As-hyperaccumulator: i) the plants’ roots should be able to extract As from soil, ii) the shoots should be capable of translocation and storage of As for easy removal, and iii) the plant must tolerate excess metal stress without adverse
impact on its own physiology (Xie et al. 2009). A suitable As-hyperaccumulator plant may find future application for remediation of As-contaminated soil and water.

*Pteris vittata* L. (Chinese brake fern) is an efficient As hyperaccumulator, and accumulates As up to 2.3% of the dry weight in its aboveground biomass (Ma et al. 2001). Discovery of this first-known As hyperaccumulator stimulated world-wide research on mechanisms by which it takes up, transports, and stores As in its vacuoles. This plant is a desirable hyperaccumulator because its aboveground biomass can store up to 93% of the total As taken up by the plant (Xie et al. 2009). Older fronds have been found with As concentrations 142 times greater than that of the soil. The gametophytes of *P. vittata* are also capable of accumulating as much as 2.5% As of their dry weight (Gumaelius et al. 2004). The plant and its gametophyte thus have a unique capability for normal physiological functions, even with such high quantities of toxic elements in their biomass.

The most common forms of As found in soil environment are As(V) and As(III). Arsenite dominates in anaerobic environments and As(V) is found in aerobic soils (Zhao et al. 2009 and Tsai et al. 2009). Arsenate is a structural analog of P and is acquired by substrate competition through phosphate transporters in plants. Arsenite is taken up into the plant by aquaglyceroporin transporters (Ali et al. 2009). Research shows that As(V) dominates in the roots whereas 70-90% of As present in fronds is As(III) in *P. vittata*. Under low P conditions typical of natural soils, As(V) is taken up more easily than As(III) by the plant roots. But As(III) dominates in the xylem sap and in the frond in *P. vittata*. There is still some uncertainty regarding the site of As(V) reduction to As(III), though arsenate reductase was found only in the roots (Duan et al. 2005). Although As(V) may
be taken up preferentially compared to As(III), As(III) is translocated faster than As(V) within *P. vittata* (Su et al. 2008).

Mechanisms of AsV uptake, movement through roots, and unidirectional transport to fronds are only partially understood. There are at least three possible mechanisms underlying As movement inside *P. vittata*: i) easy loading of As(III) into the xylem, ii) low complexation of As to the thiols, and iii) a lack of significant efflux of As from the roots (Xie et al. 2009). The precise pathway of As entry into the vacuoles and further detoxification inside the cells is also unknown, but *P. vittata* does not employ As methylation, nor volatilization for protection from As stress (Xie et al. 2009). Plants generally produce phytochelatins in response to oxidative stresses but this mechanism is also not a significant way of As detoxification in *P. vittata* (Srivastava et al. 2005). Further research on the underlying mechanisms of As translocation and storage may lead to a brighter future for phytoremediation of As-contaminated by *P. vittata* and possibly other species.

**The Unique Rhizosphere of *P. vittata***

Arsenic uptake mainly occurs via the roots, so soil rhizosphere characteristics significantly affect As hyperaccumulation by *P. vittata*. The availability of plant nutrients and As are partially controlled by the microbial community in the rhizosphere of *P. vittata*. The beneficial microorganisms include plant growth promoting rhizobacteria (PGPR) and mycorrhizae fungi. The rhizosphere is a very complex, dynamic system, which has been studied in other hyperaccumulating plants.

The symbiotic relationship between PGPR and plant roots has received considerable attention. Plant root exudates provide nutrition to rhizosphere microbes, thus increasing microbiological activity in the rhizosphere (Denton 2007). This in turn
stimulates plant growth. Microbial cells can produce and sense signal molecules, facilitating optimal timing of biofilm production by the collective. This occurs when a particular population density is achieved. The phenomenon is known as quorum sensing, which, in combination with other regulatory systems, expands the range of environmental signals that target gene expression beyond population density (Denton 2007).

Free-living as well as symbiotic PGPR can directly enhance plant growth by multiple means. One way is by providing bioavailable P for plant uptake. Another means is via fixing N for plant use. Still another way is by sequestering trace elements like Fe for plants, by production of natural Fe-chelating fluorochrome substances called siderophores. In addition, plant produces hormones like auxins, cytokinins and gibberellins, and lowers ethylene levels (Huang et al. 2004) to help in plant growth promotion. The use of PGPR in phytoremediation technologies is now being considered an important strategy, because addition of PGPR can aid plant growth in contaminated sites and enhance detoxification of soil (Kuffner et al. 2008). Three strains of PGPR, Pseudomonas putida UW3, Azospirillum brasilense Cd, and Enterobactor cloacae CAL2, were used in phytoremediation to promote plant growth and enhancing plant tolerance to contaminants (Kuffner et al. 2008). This plant-microbe interaction has not yet been studied relative to the rhizosphere of P. vittata; however it may well expand understanding of As tolerance by P. vittata and its capacity to extract As from soil minerals in the rhizosphere.

**Biotransformation of Arsenic by Bacteria**

Microorganisms play a major role in controlling the biogeochemistry of As in the environment. Transformation of As including oxidation, reduction, methylation and
demethylation is predominantly controlled by bacteria. The organic arsenic forms do not contribute significantly in soils as most of the microbial transformations take place between its +3 and +5 oxidation states of As species. A novel bacteria *Ectothiorhodospira* was isolated from Mono Lake, which was able to use As(III) as electron donor in the respiratory cycle. However, it was unable to oxidize As(III) under aerobic conditions and unable to grow in presence of As(III). These findings suggested the presence of strong coupling between respiratory As(V) reduction utilizing electron donors like organic compounds and H₂ that is replenished by microbial As(III) oxidation via oxidants like nirate, nitrite, or Fe(III) (Oremland and Stolz 2001 and Oremland et al. 2002).

The mechanisms of mobilization of inorganic As species into drinking water aquifer from insoluble soil minerals remains unclear. The possible mechanisms proposed for this phenomenon include i) oxidation of arsenopyrites (Chowdhury et al. 1999), ii) reduction of iron oxides (Harvey et al. 2002), and iii) exchange of phosphates in fertilizers for As(V) (Oremland and Stolz 2003). There is an immediate need for research to understand the process of As solubilization from insoluble soil minerals as it can leach into the ground water aquifer and cause health concerns. This may also provide us with a better understanding about the role of soil bacteria in controlling the As cycle in the environment. This knowledge will help us to make more effective use of *P. vittata*, which is less effective in accumulating As from Fe/Al minerals in the soil. In future we may be able to use these microorganisms to solubilize As from insoluble minerals and make it more available to *P. vittata*. 
Siderophores

Iron is a trace element essential for almost all living organisms including plants, animals and microorganisms. Even though Fe is the most abundant elements in the earth’s crust its availability is limited in soil environment especially in calcareous soils. This is due to the low solubility of Fe-oxide minerals in soil. Although Fe is required by plants in small amounts, Fe nutrition is critical as it is involved in many physiological processes in plants like chlorophyll synthesis, and functions as cofactor for certain enzyme functions. Iron is found in the iron-containing (heme) proteins in plants, like cytochromes that help in the electron transfer systems in chloroplasts and mitochondria. Iron is also associated with certain non-heme proteins such as ferredoxin. Most bacteria and plants have developed their own Fe acquisition mechanisms to overcome Fe limitations in soils (Winkelmann et al. 1992).

Siderophores are low molecular weight high affinity Fe-chelators produced by plants and bacteria under Fe-limiting conditions for sequestering Fe in soils. These chelators possess high specificity for Fe$^{3+}$ and the process constitutes of the following steps: 1) production siderophores inside the host cell, 2) its release outside the host, 3) complexation or dissolution of Fe-bearing minerals, and 4) recognition and uptake of Fe-siderophore complex into the host cell (Kraemer 2004). Fluorescent bacteria in soils mostly belong to the genus *Pseudomonas* sp. This ability of bacteria to sequester micronutrient by producing siderophores gives them advantage over the non-siderophore producing bacteria to survive under Fe-limiting soil conditions (Huang et al. 2004). In addition, these siderophore may also release other ions like phosphate and As(V) that are complexed with the Fe in the minerals and hence be available for plant uptake.
**Phosphorus Availability and Solubilization**

Phosphorus is a major nutrient essential for growth and development of all living organisms including plants. Total amount of P in soil is around 400-1200 mg kg\(^{-1}\) but most of it is insoluble. The concentration of soluble P in soil is only around 1 mg kg\(^{-1}\). Phosphorus reserve in soil includes both mineral and organic forms. Usually, agricultural soils accumulate large P reserves from application of inorganic P fertilizers. However, most of the soluble P from the fertilizers gets immobilized soon after application and is unavailable to crop plants. In soil, soil type and pH determine the availability of P. In acidic soils P mostly gets immobilized by iron and aluminum oxides and hydroxides while in alkaline soils it is immobilized by calcium minerals. Contribution of organic P in soil may range from 5% to as high as 95%. Inositol phosphate (phytate) constitutes around 50% of the total organic P in soil. Mostly soil microflora and plants store P as phytate, so it is the most insoluble and stable form of organic P (Rodriguez and Fraga, 1999).

In soils, both plants and microorganisms can extract P from inorganic and organic reserves by producing organic acids and phosphatase enzymes. PGPR like *Pseudomonas*, *Bacillus*, *Burkholderia* etc. associated with the rhizosphere have the ability to solubilize P and make it more available to the plant (Rodriguez and Fraga, 1999). Most microorganisms secrete organic acids, which may be able to solubilize P from insoluble soil minerals.

From the above discussion we can say that fluorescent rhizobacteria surviving in the rhizosphere of *P. vittata* may provide an important advantage to the plant in surviving under high As stress. So it is important to determine properties of the rhizosphere soil of *P. vittata* and identify the beneficial bacteria living in the rhizosphere.
The most important role of PGPR is to make nutrients more available to the plant so that it may thrive better.
CHAPTER 2
BACTERIAL ISOLATION AND CHARACTERIZATION FROM RHIZOSPHERE OF
PTERIS VITTATA

The arsenic-hyperaccumulator *Pteris vittata* is capable of extracting As from both soluble and insoluble forms (Tu et al. 2004a), and has been utilized in field applications to remediate As-contaminated soils (Shelmerdine et al. 2009; Kertulis-Tartar et al. 2006) and groundwater (Natarajan et al. 2009). Arsenic uptake in *P. vittata* mainly occurs via the roots, so soil rhizosphere characteristics significantly affect its As accumulation. *P. vittata* prefers to grow in moist shady locations where soil pH is in neutral to alkaline range. To begin with this study we located *P. vittata* growing naturally in various locations near Gainesville, Florida. We measured the soil properties and arsenic transformation rates of individual soil aliquots from each location.

The availability of nutrients and As are likely impacted by the microbial community in the rhizosphere of *P. vittata*. Free-living as well as symbiotic plant growth promoting rhizobacteria (PGPR) can enhance plant growth directly by providing available P for plant uptake, lowering plant ethylene levels, and sequestering Fe for plants by producing Fe chelating substances (Huang et al. 2004). Although some bacteria associated with *P. vittata* have been identified but they have been characterized only to a limited extent (Huang et al. 2010). We isolated arsenic-resistant bacteria from the rhizosphere soil of the arsenic-hyperaccumulating fern *P. vittata* where we found most of the arsenic was concentrated. These bacteria have to tolerate high arsenic gradient like *P. vitatta* as they reside on the roots and help the plant to survive under low nutrient soil conditions. We characterized the siderophores produced by the seven arsenic-resistant bacteria (ARB) as almost all of them were fluorescent.
Materials and Methods

Soil Collection and Characterization

The soil samples were collected from Archer Feed store (AF), Archer Ministorage (AM), Rainbow Springs (RS) and Crystal River Quarries (CQ). The sites AF and AM are located in Archer, FL, and RS site in Rainbow Springs State Park, Dunnellon, FL, and CQ site in Crystal River Quarries, FL, which is a dolomite mining site. *P. vittata* in the AF site grew under an arsenic-treated wood structure (Figure 2-1A). During rain the As may be washed out of the treated wood (Chirenje et al. 2003), which may be the source of As in this site. The AM site is close to an area, which had a previous history of As contamination (Jang et al. 2002). The RS site is located in a park and has no record of arsenic pollution, but *P. vittata* grew very close to an old wooden bridge. So this may be another example of arsenic pollution from arsenic-treated wood. From that perspective, the site CQ is unique. The fern grew on the shady slopes of the mine tailings at the edge of an abandoned open-cut dolomite mining site (Figure 2-1B). The soil samples were collected from the surface 4–5 in. from the rhizosphere of *P. vittata* and from bulk soil, which was 10 feet away from the plant where no other plants were growing. The soil samples were stored in a refrigerator before use.

Soil pH Measurement

Soil pH was measured after mixing the soil with water at 1:10 (w/w) for 2 d. Rhizosphere and bulk soil were evaluated for water-soluble As and Fe in 1:4 soil to water mixtures obtained after shaking for 1 h, centrifugation at 3500g for 15 min, and filtration through a 0.45 lm membrane filter. Arsenic in solution was determined by graphite furnace atomic absorption spectrophotometry (GFAAS, Varian AA240Z, Walnut Creek, CA). Soil samples were digested using a modified EPA Method 3050B for the
Hot Block Digestion System (Environmental Express, Mt. Pleasant, SC) and As was determined using GFAAS via EPA Method SW-846 (Tu et al. 2004a).

**Arsenic Transformation in Soils**

Soil samples (0.2 g; rhizosphere and bulk), which were stored in a refrigerator for a week prior to incubation with 3 ml of half-strength Luria Bertani medium with 2 mM glucose (modified LB) containing either 1 mM As(V) or 1 mM As(III). The bacterial medium components were purchased from Sigma Chemical Co. (St. Louis, Missouri). Sodium arsenate Na$_2$HAsO$_4$·7H$_2$O and sodium arsenite (NaAsO$_2$) were from Mallinckrodt Baker Inc. (Phillipsburg, New Jersey). The suspensions were incubated for 24 h at 30°C shaking at 200 rpm. The supernatants were analyzed for total As and for speciation of As(V) and As(III) after separation using the Sep-Pak® Plus C18 arsenic-cartridge (Water Corporation, Milford, MA; Srivastava et al. 2006). Concentrations of As(V) and As(III) were determined using GFAAS.

**Isolation and Sequencing of 16S rRNA of Arsenic-resistant Bacteria**

DNA Mr marker, Taq polymerase, dNTPs and pCR 2.1-TOPO cloning kit were from Invitrogen (Carlsbad, California). Oligonucleotide primers were synthesized using the custom primer synthesis unit of IDT DNA Technologies (Coralville, Iowa). Soil (1 g) was incubated with 10 ml 0.9% (w/v) saline solution containing 10 mM of As(V) for 24 h at 30°C shaking at 200 rpm. To isolate ARB, 0.2 ml of the saline solution was plated on modified LB agar containing half-strength LB with 2 mM glucose at pH 7 containing 10 mM As(V). Individual colonies were isolated after five rounds of single colony purification.
Genomic DNA was isolated from the bacterial isolates using the cetyltrimethylammonium bromide technique (Sambrook et al., 1989). PCR reactions, 50 µL total, contained 10 ng of genomic DNA from the bacterial strains, 20 pmol PCR primer, 40 n mole dNTPs, 125 nmol MgCl2, 250 µg bovine serum albumin, and 1 unit Taq polymerase in 1X PCR buffer (Invitrogen). The primers were ribo1492R 5' - GGTTACCTTGTTACGACTT-OH-3' and ribo27F 5' - AGAGTTTGATCCTGGCTCAG-OH-3' (Gremion et al. 2003). 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 PCR product was cloned into a pCR 2.1-TOPO vector and sequenced (Sambrook et al. 1989).

Cloned PCR products from a total of seven ARBs were sequenced. The 16S rRNA coding DNA sequences determined in this study have been deposited in GenBank (Table 2). Multiple sequence of 16S rRNA DNA was compared with sequences available in the GenBank database using BLAST (Alstchul et al., 1990).

**Thin Layer Chromatography of Siderophores**

To test the presence of siderophores in spent growth medium, the medium was extracted with chloroform. The water soluble fraction containing the siderophores was dried by injecting air through a nitrogen evaporator (N-EVAP™, Organomation Associates Inc., MA, USA) to increase the concentration of siderophores in the solution. Then the concentrated extract was analyzed using thin layer chromatography (TLC) on cellulose plates developed in a solvent mixture of butanol: acetic acid: water (3:1:2 v/v) system. To test for the presence of pyochelin type siderophores, the fluorescent spots on the TLC were sprayed with 0.1 M FeCl₃/HCl solution. Thin layer chromatography
revealed similar spots for all of the seven bacteria. So, we used Chrome Azurol S assay to quantify the amount of siderophores produced by the ARB.

**Siderophore Quantification**

We used the method of Alexander et al. (1991) to measure in vitro siderophore production. The bacterial cells were grown in 1 ml modified M9 solution at 30°C for 24 h. Modified M9 medium had the same composition as CAS medium (Schwyn and Neilands, 1987) with 5 mM MES-KOH buffer at pH 6.8. Addition of FeCl₃.6H₂O (10 µM) chelated to EDTA changed the color. After the cultures had grown to specific density determined by OD600 value measured on UV-Vis spectrophotometer, the cells were pelleted by centrifugation (10,000 g for 5 mins) and the supernatant was filtered through 0.2 micron membrane filter. The concentration of the siderophore in the filtrate was measured by mixing 500 µL of modified chrome azurol S (CAS) assay solution with 500 µL of filtrate. The standard solutions of deferroxamine mesylate were used to do the quantification assay. Zero absorbance was calibrated with a mixture of modified CAS assay solution and 1.5 mM deferroxamine mesylate. Sterile modified M9 solution was used as a reference solution, which did not contain siderophores. A standard curve was prepared by analyzing the absorbance (630 nm) of the reference solution (A/Aref) as a function of the siderophore concentration.

One of the ARB strains, i.e., PG4 (*Comamonas* sp.) did not grow in the modified M9 medium so we were unable to quantify its siderophore production. For this bacterial strain we just measured intensity of the fluorescence on the UV bench.
Results and Discussion

Soil Characterization

The four soils contained different levels of total As (Table 2-1). The RS soil contained the highest As at 235 mg kg\(^{-1}\), followed by CQ, AM and AF soils (46.3–4.42 mg kg\(^{-1}\)). In comparison, the bulk soil, taken 10 feet away from the plant, had very low As concentrations (0.07–4.07 mg kg\(^{-1}\)). It is possible that *P. vittata* may preferably grow in areas with high As concentration or the presence of *P. vittata* helped concentrate As near the rhizosphere.

The water-soluble Fe content in the rhizosphere of CQ (1.88 mg kg\(^{-1}\)) and AM (1.27 mg kg\(^{-1}\)) sites was higher than that in the corresponding bulk soils (≤0.57 mg kg\(^{-1}\)) (Table 2-1). This increase in available Fe in the rhizosphere soil may be due to the presence of Fe-solubilizing siderophore produced by rhizobacteria and root exudate by *P. vittata*. The other two soils had very low water soluble Fe (Table 2-1).

The pH in the rhizosphere soils ranged from 7 to 8, which were higher than the typical acidic soils in Florida (4.5–5.5; Chen et al. 1999). This is consistent with the fact that *P. vittata* prefers to grow in alkaline soils (Bondada and Ma 2002). The pH in the bulk soil was 6.81–7.56, but slightly lower than those in the rhizosphere (7.06–7.97; Table 2-1). This is consistent with Gonzaga et al. 2006 who observed a pH increase from 7.15 in bulk soil to 7.65 in the rhizosphere of *P. vittata* after growing for 8 weeks in arsenic-contaminated soils. At pH of 7–8, most of the nutrients become unavailable with the exception of P and As, which becomes relatively more available at this pH (Bagayoko et al. 2000).
As(III) Oxidation and As(V) Reduction in the Rhizosphere and Bulk soils

When fresh soil was incubated in modified LB medium containing 1 mM As(V) or As(III), both As(V) reduction and As(III) oxidation occurred. However, their rates differed between the rhizosphere and bulk soils and among the four soils. While both soils had high levels of As(V) reduction (67–82 mg kg⁻¹; Figure 2-2A), more As(III) oxidation occurred in the bulk soils than rhizosphere soils. This difference was most striking with AF soil, which contained 68 mg kg⁻¹ As in bulk soil compared to 4 mg kg⁻¹ As in rhizosphere soil (Figure 2-2B). This finding may indicate that more As oxidizing microbes were present in the bulk soils than in the rhizosphere soils whereas As(V) reducing microbes existed in both rhizosphere and bulk soils.

Compared to the bulk soils (0.07–4.07 mg kg⁻¹ As), As concentrations in the rhizosphere soils (4.42–235 mg kg⁻¹ As) were much greater (Table 2-1). Similar trends were observed in water-soluble As. The higher As levels in the rhizosphere of *P. vittata* may support different microflora from those in bulk soils. For typical soils, both As(V) reducing and As(III) oxidizing bacteria exist. However, in As enriched soil, bacteria with the capacity of detoxifying As(V) via reducing to As(III), the predominant form of As in soils, may be selected.

**Isolation and Identification of As-resistant Bacteria**

Three ARB were isolated from the RS and CQ soils and one from AM soil. None was obtained from the AF soil likely because the AF rhizosphere soil had the lowest total As concentration (Table 2-1) and AF bulk soil had the highest As(III) oxidation rate (Figure 2-2B). Phylogenetic analyses of the strains suggested that the strains belonged to the group gamma-proteobacteria, and three genera *Pseudomonas*, *Comamonas* and *Stenotrophomonas* sp. (Table 2-2).
Presence of Siderophores

The presence of siderophores was verified by two tests, i.e. thin layer chromatography and chrome azurol S assay.

The presence of siderophores was verified by examination of fluorescence of media extracts from the spent growth medium of the bacteria. The analysis using thin layer chromatography indicated that there were two UV-positive spots with Rf values of 0.44 and 0.00. These spots when sprayed with 0.1 M FeCl3/HCl generated orange–red coloration (data not shown), suggesting the presence of at least two compounds similar to pyochelin type siderophores (Ankenbauer et al. 1991, Cox et al. 1981).

The highest siderophore producing strains were PG12 (116 µM DFOM equiv./OD600 value of cells), PG5 (73.2 µM DFOM equiv./OD600 value of cells) and PG6 (22.6 µM DFOM equiv./OD600 value of cells). As shown in Table 2-3, the other three strains produced low amount of siderophores i.e. PG16 (15.8 µM DFOM equiv./OD600 value of cells), PG9 (10.8 µM DFOM equiv./OD600 value of cells) and PG10 (9.50 µM DFOM equiv./OD600 value of cells). The strain Comamonas sp. (PG4) failed to grow in the modified CAS assay medium.

We also related the fluorescence property of these bacterial strains to their As tolerance. We categorized them as highly fluorescent and low fluorescent. The first three bacteria PG12, PG5 and are high fluorescent bacteria while PG16, PG9 PG10 and PG6 are low fluorescent bacteria.

We found that there was significantly higher amount of As(III) oxidation by one of the bulk soil samples collected from the site AR than the bulk soils collected in other sites (Figure 2-2). Arsenate reduction was more commonly occurring in the soil aliquots collected from both the rhizosphere and bulk soil samples (Figure 2-2). These results
are consistent with that the dominant presence of As(V) reducing microorganisms in the soil samples we collected from both the rhizosphere and bulk soil samples. Both As(III) oxidizing and As(V) reducing bacteria were present in both kinds of soil samples as we recorded almost small amount of As(III) oxidation by both kinds of soil samples from the sites (MS, RS and CQ).

According to the soil properties we found that the pH in the rhizosphere of *P. vittata* was close to 7-8 (Table 2-1) and Fe is mostly insoluble in this pH range (Kraemer 2004). We know that bacteria produce Fe-chelating substances to sequester Fe for their own benefit. This natural Fe unavailability would stimulate siderophore production by the ARB and preferably such bacteria would selectively exist in alkaline pH soils. Consistent with these observations, we found that all the ARB isolated were fluorescent and producing Fe-chelating siderophores.

**Concluding Remarks**

From the above results, we concluded that the As resistant bacteria in the rhizosphere of *P. vittata* played a role in As oxidation and reduction. They may also be important in solubilizing Fe miernals as they were capable of producing siderophores. The role of rhizobacteria in releasing Fe and arsenic requires further elucidation as this may be utilized to increase arsenic uptake by *P. vittata*. 
Table 2-1. Soil pH and water soluble Fe and As, and total As concentrations (mg kg\(^{-1}\)) in four rhizosphere and bulk soils of *P. vittata*. Values are mean±SE of three replicates.

<table>
<thead>
<tr>
<th>Rhizosphere soil</th>
<th>pH</th>
<th>Total As</th>
<th>Water soluble As</th>
<th>Water soluble Fe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Archer Feed Store (AF)</td>
<td>7.97±0.09</td>
<td>4.42±0.23</td>
<td>0.50±0.34</td>
<td>0.20±0.00</td>
</tr>
<tr>
<td>Archer Ministorage (AM)</td>
<td>7.29±0.21</td>
<td>26.5±1.72</td>
<td>0.95±0.32</td>
<td>1.27±0.03</td>
</tr>
<tr>
<td>Crystal River Quarry (CQ)</td>
<td>7.06±0.04</td>
<td>46.3±1.58</td>
<td>2.50±0.39</td>
<td>1.88±0.07</td>
</tr>
<tr>
<td>Rainbow Springs (RS)</td>
<td>7.25±0.10</td>
<td>235±25.6</td>
<td>6.79±0.33</td>
<td>0.20±0.00</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Bulk soil</th>
<th>pH</th>
<th>Total As</th>
<th>Water soluble As</th>
<th>Water soluble Fe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Archer Feed Store (AF)</td>
<td>7.56±0.03</td>
<td>2.28±0.73</td>
<td>0.02±0.001</td>
<td>0.20±0.00</td>
</tr>
<tr>
<td>Archer Ministorage (AM)</td>
<td>6.81±0.04</td>
<td>4.07±0.05</td>
<td>0.12±0.004</td>
<td>0.57±0.01</td>
</tr>
<tr>
<td>Crystal River Quarry (CQ)</td>
<td>6.82±0.23</td>
<td>0.99±0.03</td>
<td>0.02±0.003</td>
<td>0.20±0.00</td>
</tr>
<tr>
<td>Rainbow Springs (RS)</td>
<td>6.92±0.03</td>
<td>0.07±0.02</td>
<td>0.01±0.001</td>
<td>0.20±0.00</td>
</tr>
</tbody>
</table>

The detection limit for As was 5 µg L\(^{-1}\) and for Fe was 0.2 mg L\(^{-1}\).
Table 2-2. Seven arsenic-resistant isolates from the rhizosphere of *P. vittata* with their specific GenBank accession numbers for 16S rRNA sequences. The nearest phylogenetic neighbor to the isolates are listed with their percentage identity. Fluorescence was scored on a UV bench at 254 nm.

<table>
<thead>
<tr>
<th>Isolate (GenBank accession number)</th>
<th>Nearest phylogenetic neighbor based on partial sequencing of 16S rRNA</th>
<th>% identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG4 (JF345176)</td>
<td><em>Comamonas testosterone</em> (CP001220.1)</td>
<td>100</td>
</tr>
<tr>
<td>PG5 (JF345177)</td>
<td><em>Pseudomonas sp.</em> (EU371554.1)</td>
<td>100</td>
</tr>
<tr>
<td>PG6 (JF345178)</td>
<td><em>Pseudomonas putida</em> (GU248219)</td>
<td>99</td>
</tr>
<tr>
<td>PG9 (JF345179)</td>
<td><em>Pseudomonas putida</em> (AM411059.1)</td>
<td>100</td>
</tr>
<tr>
<td>PG10 (JF345180)</td>
<td><em>Pseudomonas putida</em> (AY622320.1)</td>
<td>99</td>
</tr>
<tr>
<td>PG12 (JF345181)</td>
<td><em>Pseudomonas sp.</em> (FJ416144.1)</td>
<td>100</td>
</tr>
<tr>
<td>PG16 (JF345182)</td>
<td><em>Stenotrophomonas rhizophila</em> (GU186108.1)</td>
<td>97</td>
</tr>
</tbody>
</table>
Table 2-3. The amount of siderophore produced by the 6 ARB and control strain *P. chlororaphis* after incubation in 1 ml modified CAS medium for 24h at 30°C under 200 rpm measured colorimetrically based on the standard curve for Deferoxamine mesylate salt in CAS solution read at 630 nm on UV-Vis spectrophotometer. Sterile modified CAS medium was used as reference solution. The values are mean ± SE of three replicates.

<table>
<thead>
<tr>
<th>Strain Name</th>
<th>Siderophore (µM DFOM equiv.)</th>
<th>OD value of cells (A600)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG12</td>
<td>430±0.02</td>
<td>3.72±0.03</td>
</tr>
<tr>
<td>PG5</td>
<td>308±0.01</td>
<td>4.21±0.02</td>
</tr>
<tr>
<td>PG6</td>
<td>96.6±0.01</td>
<td>4.26±0.01</td>
</tr>
<tr>
<td>PG16</td>
<td>56.9±0.02</td>
<td>3.59±0.02</td>
</tr>
<tr>
<td>PG9</td>
<td>49.2±0.04</td>
<td>4.55±0.02</td>
</tr>
<tr>
<td>PG10</td>
<td>42.6±0.02</td>
<td>4.50±0.01</td>
</tr>
</tbody>
</table>

*PG4H was unable to grow in modified CAS medium.*
Figure 2-1. *P. vittata* plants growing in the Feedstore, Archer, FL site (A) and Crystal Quarry, FL site (B).
Figure 2-2. As(V) reduction (A) and As(III) oxidation (B) in four rhizosphere and bulk soils of *P. vittata*. ~0.2 g soil was mixed with 3 mL modified LB medium containing 1 mM As(V) or As(III) for 24 h. The bars represent mean ± SE. Means indicated by same letters are not significantly different at α = 0.05 using Tukey’s mean separation test.
Figure 2-3. Two different bacterial plates: one strain PG20 was slightly fluorescent (A) in the absence of As(V) and it was highly fluorescent (B) in the presence of As(V).
Arsenic is a potent poison and is mainly present as As(V) and As(III) as soluble forms in soil. Arsenate, a chemical analog of P, is taken up by living cells via P transporters (Oremland and Stolz 2003), so it interferes with phosphorylation reactions and competes with P transport (Shi et al. 2004). Similar to P, As(V) is often associated with Fe/Al minerals in soils. On the other hand, As(III) is present as a neutral species and is transported across cell membranes by glyceroporin channels (Liu et al. 2002). Arsenite reacts with the sulfhydryl groups of proteins and may inhibit many biochemical pathways. Biologically, As(III) is more reactive than As(V), as it can induce gene amplification in living cells and bind to thiol proteins (Oremland and Stolz 2003).

Both As(V) and As(III) are toxic to living organisms but some microorganisms have developed ways to survive in arsenic-rich environments. It is well known that most bacteria reduce As(V) to As(III) inside the cell through the ars operon and pass out As(III) by As(III) efflux. Arsenite can reenter cell through aquaporins and be methylated and stay within the bacterial cells (Oremland and Stolz 2003). Bacteria reduce As(V) to As(III) to minimize As(V) competition with P uptake so it can maintain normal growth and cell metabolism.

Bacterial oxidation of As(III) to As(V) has long been observed and studied (Silver and Phung 2005). Most microbial isolates lack this potential, although a range of bacteria with As(III) oxidase enzyme have been isolated and genes encoding As(III) oxidase are widely found in various groups of bacteria and archaea (Silver and Phung 2005). Periplasmic As(III) oxidase (Aox) is encoded by the two genes asoA and asoB,
which require the Fe-containing small Rieske [2Fe-2S] subunits of As(III) oxidase for their activation reported in A. faecalis (Silver and Phung 2005).

Microbial tolerance to As(V) is related to its capacity to reduce As(V) to As(III). Characterization of the anaerobic respiratory As(V) reductase enzyme showed that it also comprises of a large molybdopterin subunit, which contains an iron-sulfur center (Silver and Phung 2005). Both AsIII oxidase and anaerobic As(V) reductase require micronutrient Fe for their activation. Hence these As(III)-oxidizing and AsV-respiring bacteria occur under anaerobic low pH conditions when Fe is more available.

We have isolated and characterized seven ARB from the rhizosphere of arsenic-hyperaccumulator Pteris vittata, which grow naturally in central Florida. The rhizosphere soils contained much greater arsenic than that in bulk soils (4.42-235 vs 0.07-2.28 mg kg⁻¹). So the rhizosphere bacteria must have developed arsenic resistant system to survive in the arsenic-rich environment. Furthermore, the fluorescent ARB producing siderophores were more effective in solubilizing arsenic from insoluble Fe/AlAsO₄ minerals and increasing arsenic uptake by the plant. It is, however, unclear whether the bacteria can oxidize As(III) or reduce As(V) depending upon the arsenic species in the environment and whether their P uptake and metabolism are changed under As influence. It would be interesting to study the interaction of these bacteria with the two arsenic species As(V) and As(III).

Siderophores are fluorescent natural chelator of FeIII, which are produced by bacteria under Fe-deficient alkaline pH condition, helps sequester Fe for bacteria under aerobic conditions (Kraemer 2004). We hypothesize that this may also promote As(III) oxidation under aerobic conditions. The most commonly known soil bacteria producing
siderophores include Pseudomonads, which produce mostly pyochelin or pyoverdine type of siderophores (Kraemer 2004). The bacteria used for our study were arsenic-resistant Pseudomands, which may possess periplasmic As(III) oxidase to minimize the entry of AsIII through the aquaporins and expel As(V) out of the cells. Similarly under aerobic conditions bacteria can reduce As(V) to As(III) via ArsC and pump it out of the cells through As(III) efflux pump.

The main objective of this study was to assess the tolerance of seven arsenic-resistant bacteria to both As(V) and As(III) with control strain *Pseudomonas chlororaphis* 63-28. Previous published literature shows evidence of environmental bacteria, which are capable of only As(V) reduction under aerobic conditions and/or As(III) oxidation under anaerobic conditions. We also assessed the influence of nutrients like Fe and P on the relative rates of As(III) oxidation and As(V) reduction, which help in efflux of As(V) or As(III) out of the bacterial cells.

**Materials and Methods**

**Arsenic Transformation by Bacteria**

All ARB plus the control bacterium were incubated with 1 mM As(V) or 1 mM As(III) in modified LB medium (described in Appendix A) for at 30°C shaking at 200 rpm for 24 h. Sodium arsenate (Na₂HAsO₄·7H₂O) and sodium arsenite (NaAsO₂) salts were used for this study. The supernatants were analyzed for total arsenic and As(V) and As(III) were separated using the Sep-Pak® Plus C18 arsenic-cartridge (Srivastava et al. 2006). Concentrations of As(V) and As(III) were determined by graphite furnace atomic absorption spectrophotometry (GFAAS, Varian AA240Z, Walnut Creek, CA).
Competitive P and As(V) Uptake by Bacteria

Two ARB, PG5 and PG16, were selected based on their difference in siderophore production and As tolerance. PG5 was highly fluorescent and highly As(III) tolerant while PG16 was highly As(V) tolerant but with low fluorescent ability (see chapter 2). Control bacterium *P. chlororaphis* 63-28 is a moderately fluorescent and moderately arsenic tolerant strain (Paulitz et al. 2000). Bacterial growth, As(V) reduction rates and P utilization rates were measured in 1 ml modified LB medium containing 0, 0.1 and 1 mM As(V) along with 0.1 and 1 mM P at 0, 4, 8 and 12 h time intervals. To maintain similar concentration of all other nutrients in the 1 and 0.1 mM P strength media, modified LB growth media with 1 mM P was prepared and then diluted 10 times to obtain 0.1 mM P concentration in the media. Then 0.9 mM KH₂PO₄ salt solution was added to the diluted solution to raise the P concentration to 1 mM. Phosphate concentration was measured by modified molybdenum blue method. Bacterial growth and arsenic speciation were measured as described before.

In the transformation experiment, we used modified LB medium with a P concentration of 1 mM to grow the bacteria under 0, 0.1 and 1 mM As(V). In addition to the rate of As(V) reduction and bacterial growth, we also measured phosphorus utilization (PUI) by bacteria. The PUI was calculated by P utilized (changes in P concentration before and after experiment) by the unit dry bacterial biomass corresponding to the units OD of growth. To determine the conversion factor between OD value of cells and dry weight of biomass, 50 ml cell cultures were grown, then centrifuged down and the cell pellets were dried for 2 d at 37°C.

To measure the amount of arsenic immobilized in the cell biomass, the arsenic concentration remaining in the media after bacterial growth was measured per OD
bacterial cells. Here we used the conversion factor between the cell OD value and their dry weight. The bacterial cells were centrifuged down and washed again with fresh modified LB media to remove any arsenic immobilized on the outer surface of the cells.

**Arsenic Tolerance and Transformation by *Pseudomonas fluorescens* Pf-5**

To test the role of siderophores in arsenic tolerance and metabolism, the wild type (LK099) and two mutants of *Pseudomonas fluorescens* Pf-5, JL4900 (∆pvdI∆pchC) and LK032 (∆pchA∆pvdI) (Hartney et al. 2011) were evaluated using methods previously described.

Arsenic tolerance of all three strains was measured as percentage of growth reduction compared at 0, 1 and 10 mM of As(V) or As(III) in modified LB medium incubated at 30°C for 24 h shaking at 200 rpm. Growth was measured as absorbance at 600 nm on Beckman UV-Vis spectrophotometer.

**Statistical Analysis**

All bacterial experiments were conducted with three replicates for each treatment and every experiment was repeated at least twice. The analysis of variance (ANOVA) and Tukey’s mean grouping were used to determine significance of the interactions between the treatment means. All statistical analyses were performed with SAS statistical software (SAS Inst., Cary, North Carolina, USA).

**Results and Discussion**

The 6 ARB were separated into two groups based on their fluorescent ability: low-fluorescent (PG6R, PG9R, PG10R and PG16R) and high-fluorescent (PG5O and PG12O). We found that the low fluorescent strains were better reducers so we have used the symbol ‘R’ after them and ‘O’ after high-fluorescent strains because they were better oxidizers.
Bacterial As Tolerance was Related to As Transformation

All seven arsenic-resistant bacteria were tolerant to 1 mM As(V) with no significant reduction in growth after 24 h (Figure 3-3). However, the growth of the control bacterium *P. chlororaphis* was decreased by 18%, indicating that it was less tolerant than the 7 ARB. This may be due to the fact that metal tolerant plants like As-hyperaccumulator *P. vittata* foster a microbial community that is also metal tolerant as most of the metal uptake takes place via the roots where the rhizosphere bacteria are thriving (Sessitsch and Puschenreiter 2008). A study conducted on the functional gene diversity in the As-contaminated rhizosphere of *P. vittata* also showed that the fern maintained a significant variety of microorganisms in its rhizosphere compared to only As tolerant convergent microbial community found in typical As contaminated soil (Xiong et al. 2010). This may be due to the continuous solubilizing and uptake activity taking place by the rhizobacteria in the rhizosphere of *P. vittata* (Xiong et al. 2010).

When the bacteria were exposed to 10 mM As(V), only three low-fluorescent ARB (PG9R, PG10R and PG16R) were tolerant with no significant growth reduction. Significant growth reduction occurred for the two high-fluorescent ARB, i.e., PG5O (52%) and PG12O (10%), and *P. chlororaphis* (26%) (Figure 3-3). Under aerobic experimental conditions in our experiment, As(V) may have been reduced to As(III) by the ars operon, which does not require Fe for activation unlike the Fe-dependent activation of As(III) oxidase observed in bacteria (Silver and Phung, 2005).

However, the high-fluorescent bacteria showed higher tolerance to As(III) than the low-fluorescent ARB (Figure 3-3). The control strain *P. chlororaphis* was the least tolerant to As(III) as its growth was reduced by 68%. The strains PG5R and PG12R were the most tolerant to As(III) as the growth reduction was less than 30% and did not
reduce with increasing As(III) concentration (10 mM). For the low-fluorescent strains PG9O, PG10O, and PG16O, the growth reduction was between 20-29% in 1 mM As(III), and there was 42-50% less growth in 10 mM As(III) treatment (Figure 3-4). The control strain *P. chlororaphis* isolated from a non arsenic-contaminated area showed 68-76% growth reduction in the As(III) treatment. Even though this strain was fairly tolerant to As(V), it could not tolerate As(III) as efficiently as the ARB from the arsenic-rich rhizosphere of *P. vittata*.

To better understand bacterial arsenic tolerance mechanisms, their As(V) reduction and As(III) oxidation capability were tested at 1 mM As(V) or As(III). The low-fluorescent ARB were more effective in reducing As(V) to As(III), i.e., PG9R (65%), PG10R (58%), and PG16R (95%) compared to the high-fluorescent strains of PG5O (7.5%) and PG12O (22.5%). But the high-fluorescent ARB were more effective in oxidizing As(III) to As(V), i.e., PG5 (46%) and PG12 (31%) compared to low-fluorescent ARB, i.e., PG9 (21%), PG10 (6.2%) and PG16 (14%) (Figure 3-2). The bacterial strain PG6R, which produced 96.6 µM equiv. of siderophores was capable of 63% As(V) reduction and 19% As(III) oxidation. The control bacterial strain *P. chlororaphis* reduced 100% As(V) to As(III) in 24 h but did not oxidize As(III) to As(V).

Arsenic-resistant bacteria can expel arsenic out of the cells utilizing the ars operon after its reduction from As(V) to As(III). This process will help bacteria to keep arsenic out of the cell when As(V) is the dominant species in the medium but after certain time all the As(V) will be reduced to As(III). So, it is also important for ARB to deal with As(III) entering through the aquaporins. Oxidation of As(III) to As(V) by the periplasmic As(III) oxidase would balance the other half of the transformation of As(V) to
As(III) and vice versa. The high-fluorescent strains PG5R (308 µM equiv.) and PG12R (430 µM equiv.) produced the highest amount of siderophore (Table 3-2). Though *P. chlororaphis* was an As(V) reducing bacterium, its siderophore production was low at 68 µM equiv. (Table 2-3).

The high fluorescent bacterial strains, which were good As(III) oxidizers, retained less arsenic when exposed to 1 mM As(III) and the bacterial strains that were better reducers retained less arsenic in the 1 mM As(V) treatment. Bacterial strain PG5 retained 105% more arsenic in the As(V) treatment compared to As(III) treatment (Figure 3-4) and was highly tolerant to As(III) (Figure 3-2) and least tolerant to As(V) among all the strains (Figure 3-1). PG12R retained only 30% more arsenic in the As(V) treatment compared to the As(III) treatment (Figure 3-4) as it had almost equal rates of As(III) and As(V) oxidation and reduction (Figure 3-3), and was equally tolerant to both As(III) and As(V) (Figure 3-1 and 3-2). The bacterial strain PG10O that produced only 43 µM equiv. siderophore (Table 2-3) retained 63% more arsenic in the As(III) compared to the As(V) treatment (Figure 3-4), was highly tolerant to As(V) and 40% less tolerant to As(III) compared to As(V) (Figure 3-2). Control strain was highly efficient in reducing 1 mM As(V) to As(III) but it was unable to oxidize As(III) to As(V) (Figure 3-3) and this strain was not tolerant to As(III). *P. chlororaphis* reduced growth by ~20% in 1 mM As(V) treatment (Figure 3-1) vs. 70% growth reduction under 1 mM As(III) treatment (Figure 3-2).

Arsenate reduction and As(III) oxidation helped in expelling As out of the cell, enhancing As tolerance in As-resistant bacteria. Siderophore producing fluorescent bacteria were capable of higher rate of As(III) oxidation capacity ($r=0.77$, $p=0.01$),
whereas there was inverse relationship with the rate of As(V) reduction ($r = -0.81$, $p=0.01$). Siderophile producing fluorescent bacteria were also more tolerant to As(III) ($r = 0.62$, $p=0.01$).

**Influence of P on Bacterial As(V) Reduction**

The major route of As(V) entry into a bacterial cell is via P transporters. So we studied the P/As ratio on bacterial arsenic reduction. Phosphate uptake occurred during the first 4 h where there was minimum bacterial growth. Addition of arsenic (0.1-1 mM) to the media containing 1 mM P significantly increased P uptake during the first 4 h. We hypothesize that this increase was due to arsenic-induced P uptake via upregulation of P transporters, which was supported by phosphorus utilization (PUI) (Table 3-1). Take PG16O for example, its PUI increased 1.6 and 3.0 times when it was exposed to 0.1 and 1 mM As(V). In case of the control bacterium, its PUI increased 3.0 and 8.4 times. The high-fluorescent ARB PG5O was remarkable because its PUI was decreased by 2.8 times at 0.1 mM As(V) and increased by 1.5 times at 1 mM As(V). The two ARB showed less arsenic-induced P uptake compared to *P. chlororaphis* as they were isolated from arsenic-contaminated soil and they may have adapted themselves to utilize less P to grow. In soil the typical available P is low at ~1 ppm (Rodriguez and Fraga 1999). In the rhizosphere of arsenic-hyperaccumulating fern *P. vittata* the ARB need to survive under high As and low P condition. So the ARB, which takes up less P under high arsenic conditions, has higher chances of surviving under natural conditions in the rhizosphere of *P. vittata* where P is limiting.

The control bacterium, which reduced 72% of 1 mM As(V) after 12 h in modified LB medium, also showed the highest PUI of 39 vs. 4.6 mg g$^{-1}$ dw under no arsenic condition. This supports the hypothesis that As(V) upregulated P transporters, resulting
in more P uptake by the bacterium. PG16R, As(V) reducing strain showed significantly lower value of PUI and higher rate of 89% As(V) reduction with 1 mM As(V) (Table 3-1). P utilization rate and As(V) reduction by bacteria were inversely correlated. The bacterial strain PG16O showed only 14% oxidation of As(III) to As(V) at 1 mM As(III) after 24 h incubation (Figure 3-3). PG5R showed the lowest PUI as well as the lowest rate of only 47% of As(V) reduction after 12 h. This bacterium was primarily an As(III) oxidizer as it showed 46% As(III) oxidation at 1 mM As(III). This bacterium was also producing significantly high amounts of siderophores (308 µM equiv.).

**Arsenic-resistant Bacteria Retained Less As in the Biomass**

The amount of As(V) and As(III) immobilized in the cell biomass may be related to their arsenic tolerance. As As(V) tolerance by bacteria is through its uptake, reduction and subsequently expulsion out of the cells (Lloyd and Oremland 2006), lower As(V) may imply better arsenic tolerance. Arsenite after getting into the cells requires to be expelled out, otherwise would complex with the thiol group of the proteins and be immobilized in the cells. Hence, As(III) oxidation by the As(III) oxidizing bacteria is a detoxification mechanism by microorganisms (Liu et al. 2002, Chang et al. 2007) as it expels As(V) out of the cell similar to As(III) efflux pump of ars operon. Among the 6 ARB, the low-fluorescent bacteria PG9O (49.2 µM), 10O (42.6 µM) and 16O (56.9 µM), which were the most tolerant to AsV (Figure 3-1), low siderophore producer (Table 2-3) and strong As(V) reducers (Figure 3-4), retained the least amount of As after exposing to 1 mM As(V).

To better understand the interaction of P and As(V), we repeated the experiment at 1 As(V) and 0.1 or 1 mM P using PG5O and PG12O. From Table 3-2 the arsenic uptake was significantly less in 1 mM P treatment for *P. chlororaphis* and PG16R
bacterial strains. After 12 h of incubation, in all three strains significantly less reduction of As(V) to As(III) at 1 mM P compared to 0.1 mM P treatment due to competition in P and As(V) uptake. This slower rate of As(V) reduction also resulted in retaining more arsenic in the bacterial biomass at 1 mM P compared to the 0.1 mM P treatment (Table 3-2). This also proves that As(V) reducing bacteria retained less arsenic under low P natural conditions existing in soil.

For bacterial tolerance to As(III), they can eliminate more arsenic through As(III) oxidation compared to the bacteria that were unable to effectively oxidize As(III). As a group, PG5O and 12O were slightly more tolerant to As(III) than PG6R, 9R,10R and 16R though the separation was not as clear as As(V) tolerance. The more tolerant group was also more efficient in siderophore production and As(III) oxidation (Table 2-3). In terms of As(III) in cell biomass, except for PG4R, they were also lower compared to the As content in the biomass in the As(V) treatment. It is interesting to note that all 7 As-resistant bacteria were capable of both As(V) reduction and As(III) oxidation under aerobic culture conditions. This is probably because the speciation of bioavailable inorganic As species in soil is controlled by biotransformation and it keeps changing between As(V) and As(III) (Lloyd and Oremland 2006). When bacteria are exposed to metal oxidative stress, reactive oxygen species are produced. Previous studies on arsenic-resistant bacteria isolated from the rhizosphere of *P. vittata* have shown that these bacteria may be more effective in producing antioxidant enzymes such as super oxide dismutase and catalase that help them in scavenging the reactive oxygen species produced due to As stress (Huang et al. 2010). Another study conducted on *P. aeruginosa*, showed that mutants lacking several functional genes like ArsB As(III)
membrane pump, glutathione reductase or super oxide dismutase were less tolerant to As(III) compared to the wild type (Parvatiyar et al. 2005). Several gene operons influence metal like As, Cd tolerance and homeostasis have been found in P. putida that also belongs to the broad genus of bacteria we are studying for As tolerance and P uptake (Canovas et al. 2003, Wu et al. 2011). So, the bacteria surviving in arsenic-rich environment need to be tolerant to both As(V) and As(III), which may be a unique feature of ARB. However, this was not the case with the control bacterium, which was only effective in As(V) reduction.

The As(III) oxidation and As(V) reduction helps bacteria in expelling As out of their cells. Arsenate entering the cell is reduced to As(III) and expelled out through As(III) efflux. Previous study conducted on P. pseudomallei with radiolabelled As(III) showed that As(III) tolerance is correlated with retention of less amount of As(III) in the bacteria cells during their growth process (Beppu and Arima 1964). This process may help detoxification of As(V) because As(V) is a structural analog of P and in the presence of As(V), the PUI increased from 2-209% in 0.1 mM As(V) treatment and 49-740% in 1 mM As(V) treatment in all bacteria (Table 3-1). This phenomenon was observed in the first 4 h of bacterial incubation during the phase when ~90% of the P present in the growth medium was taken up by all three bacteria. So, we can say that in the presence of As, bacteria increased P uptake. Similar to As(V), As(III) is also highly toxic to the cells and enters cells through aquaporins (Silver and Phung 2005). Arsenite oxidase (Aox) is located in the periplasm of bacterial cells and can expel As(V) out of the cell after oxidizing As(III) to As(V). However, Fe-S complex is required for the activation of AOX, so the bacteria producing more siderophores may be more efficient
in acquiring Fe(III) and in As(III) oxidation. Hence, As(III) oxidizing bacteria retain less As in 1 mM As(III) treatment and As(V) reducing bacteria retain less As in 1 mM As(V) treatment (Figure 3-4).

**Arsenic Tolerance in *Pseudomonas fluorescens* Mutants**

To link siderophore production with arsenic tolerance, we tested the arsenic tolerance of two Pf5 mutants (*pchC* and *pchA*) with impaired siderophore production ability (Hartney et al. 2011). The *pchC* gene construct was made by using GmR-gfp cassette to interrupt the *pchC* gene. The *pchA* gene construct was made by deleting the middle portion of the *pchA* gene. The pchC mutant was able to produce residual amount of enantio-pyochelin siderophores and the pchA mutant did not produce any siderophore (Hartney et al. 2011). When exposed to 1 mM As(V), the two mutants were 16% less tolerant to 1 mM As(V) than the wildtype (Figure 3-5A). However, this was not the case with 1 mM As(III) where *pchA* was 23% less tolerant while *pchC* showed no difference with the wildtype (Figure 3-5B). At 10 mM As(III), both mutants and the wild type were inhibited to similar extent (Figure 3-5B) suggesting that siderophore production’s positive correlation to As tolerance depend on both the As species and concentration.

**Concluding Remarks**

The increased P uptake in the presence of As(V) helped in decreasing arsenic toxicity in bacteria surviving in low-P available and arsenic-rich soil environments. Arsenic resistant bacteria have developed the ability to reduce As(V) to As(III) inside the cells and release As(III) so that it does not interfere with P uptake. This biotransformation of As(V) to As(III) also led to the As(III) accumulation. However, some of ARB oxidized As(III) back to As(V). Together As(V) oxidizing and As(III)
reducing bacteria in soil could reduce the inhibitory effects of arsenic to cellular metabolism.
Table 3-1. Phosphate utilization index (PUI) of three bacterial strains PG16R (As(V) tolerant), *P. chlororaphis* (control) and PG5O (As(III) tolerant) after growing 4, 8 and 12 h in modified LB medium with 0.1 mM P and 0, 0.1 or 1 mM As(V). The values are mean ± SE of three replicates.

<table>
<thead>
<tr>
<th>Strain Name</th>
<th>Growth Time (h)</th>
<th>P utilization (PUI)* (mg g⁻¹ dw)</th>
<th>AsV reduction in media (mg L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 mM AsV</td>
<td>0.1 mM AsV</td>
</tr>
<tr>
<td>PG16R</td>
<td>4</td>
<td>0.53±0.020</td>
<td>0.86±0.005</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0.15±0.013</td>
<td>0.18±0.003</td>
</tr>
<tr>
<td></td>
<td>12**</td>
<td>0.16±0.010</td>
<td>0.17±0.003</td>
</tr>
<tr>
<td>PC</td>
<td>4</td>
<td>4.62±0.010</td>
<td>14.3±0.003</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0.21±0.005i</td>
<td>0.23±0.003i</td>
</tr>
<tr>
<td></td>
<td>12**</td>
<td>0.17±0.003</td>
<td>0.18±0.003</td>
</tr>
<tr>
<td>PG5O</td>
<td>4</td>
<td>4.58±0.010</td>
<td>4.66±0.003</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0.29±0.005j</td>
<td>0.26±0.003j</td>
</tr>
<tr>
<td></td>
<td>12**</td>
<td>0.17±0.003</td>
<td>0.18±0.003</td>
</tr>
</tbody>
</table>

We used Tukey’s mean grouping test and same letter denotes means belonging to the same group. We used ‘a’ to denote PUI and ‘A’ for As(V) reduction in media.

**Approximately 90% of the total P in the growth media was taken up by the bacterial cells within 8 h time interval.

*PUI (Phosphate Utilization) = changes in P concentration before and after experiment divided by cell biomass

PC = control bacterium *P. chlororaphis*
Table 3-2. Arsenic content (mg g\(^{-1}\) dw) and concentration of AsIII/AsV in the spent growth media after 4 and 12 h incubation with arsenic-resistant bacteria of PG5 and PG16 with control bacteria of \(P.\) chlororaphis. Different P concentrations (0.1 and 1 mM) and single As(V) concentration of 1 mM in modified LB medium. The values are mean ± SE of three replicates.

<table>
<thead>
<tr>
<th>Bacteria strain</th>
<th>Growth time (h)</th>
<th>P concentration in LB medium (mM)</th>
<th>As content (mg g(^{-1}) dw)* under different P (mM)</th>
<th>AsIII conc (mgL(^{-1}))</th>
<th>AsV conc (mgL(^{-1}))</th>
<th>AsIII conc (mgL(^{-1}))</th>
<th>AsV conc (mgL(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>P C</strong></td>
<td>4</td>
<td>6.5±1.7A</td>
<td>11.9±1.4B</td>
<td>3.22±0.8A</td>
<td>66.9±1.3a</td>
<td>1.16±0.1B</td>
<td>56.6±2.4b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.57±0.2C</td>
<td>2.45±0.3D</td>
<td>55.3±0.6C</td>
<td>18.3±0.9c</td>
<td>20.6±1.3D</td>
<td>36.6±1.4d</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>11.9±1.4B</td>
<td>66.9±1.3a</td>
<td>1.16±0.1B</td>
<td>56.6±2.4b</td>
<td>18.3±0.9c</td>
<td>36.6±1.4d</td>
</tr>
<tr>
<td><strong>PG5</strong></td>
<td>4</td>
<td>4.35±0.4E</td>
<td>3.61±0.3E</td>
<td>2.63±0.7A</td>
<td>56.7±3.6b</td>
<td>1.72±0.3E</td>
<td>52.7±0.5e</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.82±0.6D</td>
<td>3.13±0.3E</td>
<td>21.8±0.4D</td>
<td>44.1±2.5f</td>
<td>11.6±0.4F</td>
<td>43.8±2.6f</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>7.12±0.4F</td>
<td>2.10±0.2D</td>
<td>3.92±0.3A</td>
<td>65.4±4.3a</td>
<td>2.36±0.4A</td>
<td>56.7±3.5b</td>
</tr>
<tr>
<td><strong>PG16</strong></td>
<td>4</td>
<td>1.54±0.8D</td>
<td>3.80±0.1E</td>
<td>11.5±0.4F</td>
<td>58.4±2.9b</td>
<td>9.35±0.2G</td>
<td>52.9±1.8e</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>1.54±0.8D</td>
<td>3.80±0.1E</td>
<td>11.5±0.4F</td>
<td>58.4±2.9b</td>
<td>9.35±0.2G</td>
<td>52.9±1.8e</td>
</tr>
</tbody>
</table>

We used Tukey’s mean grouping test and same letter denotes means belonging to the same group. We used ‘A’ to denote As content in cell biomass and ‘A’ for As concentration in media.

*Arsenic concentration in the cell biomass of the bacteria was measured as mg g\(^{-1}\) dw. PC = control bacterium \(P.\) chlororaphis
Figure 3-1. Arsenic tolerance of 7 ARB, PG5(O), 6(O), 12(O), 9(R), 10(R), 16(R) and control *P. chlororaphis* (PC) measured as % growth reduction of the control in 1 ml modified LB medium spiked with 1 and 10 mM As(V). The bacteria were grown for 24 h at 30°C under 200 rpm. The bars represent mean ± SE of three replicates. We used Tukey’s mean grouping test and same letter denotes means belonging to the same group.
Figure 3-2. Arsenic tolerance of 7 ARB; PG5O, 6O, 12O, 9R, 10R, 16R and control strain *P. chlororaphis* (PC) measured as % growth reduction of the control in 1 ml modified LB medium spiked with 1 and 10 mM As(III). The bacteria were grown for 24 h at 30°C under 200 rpm. The bars represent mean ± SE of three replicates. We used Tukey’s mean grouping test and same letter denotes means belonging to the same group.
Figure 3-3. Arsenic oxidation and reduction by 7 ARB; PG5O, 6O, 12O, 9R, 10R, 16R and control strain *P. chlororaphis* (P.c.) in modified LB medium spiked at 1 mM As(V) or As(III). The bacteria were grown for 24 h at 30°C under 200 rpm. The control here refers to modified LB medium incubated without bacteria. The bars represent mean ± SE of three replicates. We used Tukey’s mean grouping test and same letter denotes means belonging to the same group. We have two mean groups, oxidation denoted by ‘A’ and reduction denoted by ‘a’.
Figure 3-4. Amount of arsenic immobilized in the cell biomass by the 7 ARB; PG5O, 6O, 12O, 9O, 10O, 16O and control strain *P. chlororaphis* (P.c.) in modified LB medium spiked at 1 mM AsV or AsIII. The bacteria were grown for 24 h at 30°C under 200 rpm. The bars represent mean ± SE of three replicates. We used Tukey’s mean grouping test and same letter denotes means belonging to the same group. We have two mean groups, As(V) denoted by ‘A’ and As(III) denoted by ‘a’.
Figure 3-5. Arsenic tolerance of the two mutants and wildtype of *P. fluorescens* measured as % growth reduction of the control in 1 ml modified LB medium after 24 h at 1 mM As(V) (A) and 1 mM As(III) (B). The bars represent mean ± SE of three replicates. We used Tukey’s mean grouping test and same letter denotes means belonging to the same group. We have one mean group in figure 3-5A and two mean groups in figure 3-5B, 1 mM As(III) denoted by ‘A’ and 10 mM As(III) denoted by ‘a’.

*All three strains failed to grow in 10 mM As(V)*
CHAPTER 4
ARSENIC SOLUBILIZATION BY BACTERIA AND UPTAKE BY P. VITTATA

The health hazards associated with arsenic-contaminated soil coupled with the high remediation cost makes it necessary to develop cost-effective technologies to restore polluted land. Phytoremediation is a cost-effective technique, which employs plants to remediate polluted soils and to improve soil properties. It is effective in removing metals of low concentrations from surface soils and water, although longer treatment times are required compared to other conventional techniques such as soil excavation and disposal to landfill.

The arsenic hyperaccumulator *Pteris vittata* is a viable candidate for phytoremediation of As-contaminated sites. It is capable of extracting As from both soluble and insoluble forms (Tu et al. 2004a), and has been utilized in field applications to remediate As-contaminated soils (Shelmerdine et al., 2009; Kertulis-Tartar et al. 2006) and groundwater (Natarajan et al. 2009). The availability of nutrients and As are likely impacted by the microbial community in the rhizosphere of *P. vittata*. The Fe solubilization activity of PGPR can impact As levels in soils. In aerobic soils, As(V) is present as HAsO$_4^{2-}$ and H$_2$AsO$_4^-$, and is often bound to Fe/Al minerals, making it insoluble (Tu et al. 2004a), however, Fe solubilization also releases the As from the minerals, making it bioavailable to plants and soil microorganisms.

The isolated ARB from the rhizosphere of *P. vittata* produced Fe-chelating substances siderophores (See Chapter 2). In this experiment we assessed the properties of the fluorescent ARB in solublizing Fe/Al and arsenic from insoluble soil minerals like FeAsO$_4$ and AlAsO$_4$. This experiment also elucidated the plant-microbe interaction between the arsenic-hyperaccumulating fern and the fluorescent Pseudomonads.
surviving on the roots of the plant in making both Fe and arsenic more available in the rhizosphere for better uptake by *P. vittata*.

Although some bacteria associated with *P. vittata* have been identified and characterized to a limited extent (Huang et al. 2010), their effects on As uptake have not been examined. In the current study, seven As-resistant bacteria from the rhizosphere soil were isolated and identified, and their As transformation ability to improve plant growth and As uptake via solubilizing Fe and As from insoluble minerals was determined.

**Materials and Methods**

**Arsenic Solubilization and Plant Growth by ARB**

FeAsO$_4$ and AlAsO$_4$ minerals were synthesized (Tu et al., 2004a), washed, and dried at 300°C for 3 h. Six-month old *P. vittata* plants, which were purchased from Milestone (Apopka, FL), were acclimated in 1 L of 0.25X Fe-free Hoagland medium at pH 7 for 2 weeks. Fe-free medium was used to simulate the low-Fe growth habitat of *P. vittata* in the environment (Bondada and Ma, 2002). Then 0.25 g of pre-washed FeAsO$_4$ or AlAsO$_4$ mineral was added to the medium. The seven ARB isolates were grown overnight separately and then mixed together such that equal cfu/ml of each isolate was represented in a volume of 12 ml at a density of OD600 value of 1.5. One ml of this mixture was added to 1 L of growth medium. After 7 d, arsenic concentration was measured in the plant tissue and the growth medium, and fresh plant biomass of *P. vittata* was recorded after washing the plant roots in DI water and drying on blotting paper. The plant samples were dried in an oven at 80°C for 2 d, digested with H$_2$SO$_4$/H$_2$O$_2$ (Tu and Ma 2003), and analyzed for arsenic using graphite furnace atomic
absorption spectrometry (GFAAS, Varian AA240Z, Walnut Creek, CA). In addition to arsenic, total Fe and Al concentrations in the plants were measured in the same extracts. To determine the amount of Fe/Al precipitating on root surfaces, plant roots (0.2 g) were washed in 50 mL 1:1 v/v HNO₃ and water for 20 min and Fe and Al concentrations in the supernatants and plant digest were determined using inductively-coupled plasma emission spectroscopy (PerkinElmer 5300DV, Waltham, MA) via EPA Method 2007. To confirm the role of root exudate in solubilizing As from FeAsO₄ and AlAsO₄ minerals, dissolved organic C (DOC) in the spent growth medium was analyzed using a total carbon analyzer (TOC-5050A, Shimadzu) equipped with an autosampler (Tu et al. 2004a).

**Arsenic Solubilization and Plant Growth Effects of ARB and Bacillus**

To further test the efficiency of ARB in arsenic solubilization, the experiment described in the previous section was repeated with soil bacteria Bacillus sp. (GenBank accession number JN132398) as a control, which was grown to an OD600 of 1.5. We selected Bacillus sp. as our control bacterium because it is a commonly found soil bacterium isolated from a non arsenic-contaminated soil, and there has been no report of *P. vittata* being infected by any bacterial pathogen present in soil. After 7-d of growth, plant biomass (dw) was determined after drying in an oven at 80°C for 2 d.

To determine the potential role of P in enhancing plant growth, dried plant samples were digested using a H₂SO₄/H₂O₂ to analyze P using modified molybdenum blue method. Briefly the pH of the digestion solution was adjusted to 5 with NaOH and HCl. Ten milliliter of the solution was pipetted into a 20 mL glass test tube, and to this 0.5 mL of L-cysteine (5% w/v in 0.6 M HCl) was added. The test tube was capped tightly

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to allow As(V) reduction to As(III) for 5 min at 80°C. The solution was cooled to room temperature, and P was determined by the molybdenum blue method.

To separate the effect of arsenic from that of Fe, six-month old *P. vittata* plants were acclimated in 1 L of 0.25X Fe-free Hoagland medium at pH 7 for 2 weeks. The treatment had 0 or 3 mg/L water-soluble As(V) with and without the ARB consortium. The fresh plant weight was recorded after 7 d of treatment and corresponding dry weights were measured after drying in an oven at 80 °C for 2 d.

**Results and Discussion**

**Solubilization of Arsenic from FeAsO₄ and AlAsO₄ by *P. vittata* and ARB**

Both *P. vittata* and arsenic-resistant bacteria were effective in solubilizing As from insoluble FeAsO₄ and AlAsO₄ minerals added to the hydroponic growth medium (Table 4-1). In the absence of *P. vittata* and ARB (no plant and no ARB), neither FeAsO₄ nor AlAsO₄ mineral was solubilized (As = 0.005 mg L⁻¹). In the presence of *P. vittata* (*P. vittata* with no ARB), a substantial amount of As was solubilized, with more As from AlAsO₄ (5.04 mg L⁻¹) than FeAsO₄ treatment (2.58 mg L⁻¹) being solubilized (*p* = 0.01; Table 4-1). Similarly, ARB inoculation (no plant with ARB) was effective in solubilizing As in the medium, with more from AlAsO₄ than FeAsO₄ treatment (8.02 vs. 4.14 mg L⁻¹). There were two possible reasons for higher As solubilization from AlAsO₄ than from FeAsO₄ treatment. The substantially higher solubility of AlAsO₄ than FeAsO₄ probably makes it more soluble in the medium (*Ksp* = 1.6 X 10⁻¹⁶ vs. 5.7 X 10⁻²¹; Patnaik, 2004). In addition, it is possible that, under Fe-deficient conditions in the AlAsO₄ treatment (0.25X Fe-free Hoagland solution), the plants and ARB exuded more organic acids to obtain Fe. This idea was supported by the 12–76% more DOC in the
AlAsO₄ than the FeAsO₄ containing medium (Table 4-1). *P. vittata* produced 1.84 mg L⁻¹ DOC in the FeAsO₄ compared to 3.24 mg L⁻¹ in the AlAsO₄ treatment (Table 4-1), which is consistent with the data by Tu et al. (2004a).

Both *P. vittata* and ARB were effective in solubilizing As; however, ARB was 59–60% more effective than *P. vittata* in As solubilization (Table 4-1). Though no bacteria were added to the *P. vittata* treatment, bacteria were present on the roots of *P. vittata*; however, these bacteria were not as effective as ARB in As solubilization. Inoculation of ARB in *P. vittata* treatment further increased As concentrations, especially in the AlAsO₄ treatment, which increased by 46% (Table 4-1). Significantly higher amount of As solubilized by ARB than *P. vittata* may be due to the fact that more siderophores were produced by ARB under Fe-starvation conditions to complex with Fe or Al, making As more soluble. The presence of siderophores was verified by examination of fluorescence of media extracts. The analysis using thin layer chromatography indicated that there were two UV-positive spots with Rf values of 0.44 and 0.00. These spots when sprayed with 0.1 MFeCl₃/HCl generated orange–red coloration (data not shown), suggesting the presence of at least two compounds similar to pyochelin type siderophores (Ankenbauer et al. 1991, Cox et al. 1981).

**Effect of ARB on Uptake and Translocation of As, Fe and Al by *P. vittata***

In addition to being effective in As solubilization from FeAsO₄ and AlAsO₄ minerals, *P. vittata* was also able to take up As from the growth medium (Table 4-2). However, As concentrations in the plant were low with only small amount of As being translocated from the roots to fronds, resulting in the As translocation factor (TF; concentration ratio in fronds to roots) of 0.9 in FeAsO₄ and 0.5 in AlAsO₄ treatments. Unlike As (Table 4-1), neither Fe nor Al was detected in the growth medium (data not
shown). However, *P. vittata* was able to take up some Fe and Al, with frond and root concentrations being 19.8 and 385 mg kg\(^{-1}\) Fe in FeAsO\(_4\) treatment, and 2.00 and 39.2 mg kg\(^{-1}\) Al in AlAsO\(_4\) treatment (Table 4-2). Apparently, *P. vittata* was ineffective in taking up or translocating Al or Fe (TF < 0.08; Table 7). Inoculation with ARB significantly increased As concentrations in *P. vittata* (Table 4-2). While substantially more As was accumulated in the roots than fronds (450 vs. 35.3 mg kg\(^{-1}\)) in the FeAsO\(_4\) treatment, significant amount of As was translocated from roots to fronds in *P. vittata* in AlAsO\(_4\) treatment (180 vs. 236 mg kg\(^{-1}\)). The higher amount of Fe accumulated in the roots (662 vs. 385 mg kg\(^{-1}\)) with ARB treatment may have hindered arsenic translocation in the FeAsO\(_4\) treatment. Only in AlAsO\(_4\) treatment, ARB treatment increased As translocation compared to no ARB treatment, with As TF increasing from 0.5 to 1.3. The fact that much higher Fe concentration was observed inside the roots (roots-internal; 85–95%) than the root surface (roots-external; 5–15%) is consistent with siderophore-assisted Fe uptake by *P. vittata* (Table 4-2).

**Effect of ARB and *Bacillus* sp. on Growth of *P. vittata***

Inoculation of ARB substantially increased the concentrations of As, Fe and Al in *P. vittata*, with the impacts being more significant in the roots than the fronds (Table 4-2). For example, ARB increased the arsenic concentrations by 2–11 times in the fronds compared to 4–23 times in the roots. A similar pattern was observed for *P. vittata* biomass (Table 4-3). Little change was observed in frond biomass but ARB increased root biomass by 89% in the FeAsO\(_4\) treatment and 130% in the AlAsO\(_4\) treatment. The release of Fe from the FeAsO\(_4\) + ARB treatment may have helped *P. vittata* growth; however, the increase of *P. vittata* biomass in the AlAsO\(_4\) + ARB treatment may not be due to Al uptake as it is not a nutrient. Our data clearly demonstrated that ARB was
more effective in promoting *P. vittata* growth than the microbes associated with *P. vittata* roots in the no ARB treatment (Table 4-3). To better understand the role of ARB in promoting plant growth, we repeated the experiment with bacteria *Bacillus* sp. being added as a treatment. No difference was observed between the control and *Bacillus* sp. treatment (Figure 4-1A), but ARB increased both root and frond biomass, with a larger increase being observed in the roots than the fronds and in the AlAsO$_4$ than in the FeAsO$_4$ treatment. For example, the increase in root and frond biomass was 47% and 23% in FeAsO$_4$ treatment compared to 74% and 60% in AlAsO$_4$ treatment, respectively (Figure 4-1A). Since plant biomass increase was larger in the AlAsO$_4$ treatment than that in the FeAsO$_4$ treatment (Figure 4-1A), Fe probably did not play a significant role as a nutrient. We hypothesized that arsenic-induced P uptake by *P. vittata* may have helped its growth. In a hydroponic experiment, Luongo and Ma (2005) observed that plant biomass increase after exposure to 1 mg L$^{-1}$ As for 2 weeks was associated with a substantial increase in P uptake by *P. vittata*. Compared to the control, P concentrations increased from 2.33 to 5.19 g kg$^{-1}$ in the fronds, and from 0.91 to 5.76 g kg$^{-1}$ in the roots (Luongo and Ma 2005). Similar results were obtained by Tu et al. (2004b) as well as in this experiment. It is possible that As stress may have upregulated P uptake by *P. vittata* (Figure 4-1B). For example, ARB increased root P by 45% in FeAsO$_4$ treatment and by 266% in AlAsO$_4$ treatments. The much greater increase in root P concentrations (Figure 4-1B) was consistent with greater increase in root biomass (Figure 4-1A). The fact that P concentrations and biomass in the roots ($r = 0.64$) was better correlated than that in the fronds ($r = 0.44$) ($p = 0.05$), also supported our hypothesis that arsenic-induced P uptake was probably responsible for biomass increase in *P. vittata*. To further
examine this hypothesis, the impact of As on the ability of ARB in promoting plant
growth was further tested. In this experiment, same cfu/ml of ARB consortium was
inoculated as the experiment described in materials and methods section to the growth
medium with or without 3.0 mg/L arsenic. The level of As concentration in the growth
medium was similar to the experiment discussed in materials and methods section
(Table 4-1). Consistent with our hypothesis, higher frond and root biomass were
obtained with As (4.8 and 3.5 g/fronds and roots dw, respectively) than without As (3.5
and 1.9 g/plant dw) treatments (data not shown), again supporting arsenic-induced P
uptake.

Concluding Remarks

*P. vittata* and ARB were efficient in solubilizing As from insoluble FeAsO$_4$ and
AlAsO$_4$, with ARB being more efficient than *P. vittata*. The fern was inefficient in taking
up Al, and high As and Fe in the roots suggested possible co-precipitation of arsenic
and Fe. Arsenic-resistant bacteria were efficient in promoting *P. vittata* growth. There
was dependence between *P. vittata* and the fluorescent ARB in its rhizosphere. In the
presence of ARB and As, the increase in P uptake by *P. vittata* was probably due to
arsenic-induced P deficiency, which enabled the plant to grow better in the arsenic-rich
environment. In the future, ARB may be applied in field applications to enhance As
hyperaccumulation by *P. vittata*. 
Table 4-1 Effect of arsenic-resistant bacteria (ARB) on As and dissolved organic C (DOC) concentrations in 0.25X Fe-free Hoagland medium where *P. vittata* grew for 7 d. The medium was spiked with insoluble FeAsO$_4$ and AlAsO$_4$ minerals and ARB.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>As concentrations (mg L$^{-1}$)</th>
<th>DOC concentrations (mg L$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>With ARB</td>
</tr>
<tr>
<td>FeAsO$_4$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No plant</td>
<td>0.005±0.0*A</td>
<td>4.14±0.19BC</td>
</tr>
<tr>
<td><em>P. vittata</em></td>
<td>2.58±0.39B</td>
<td>2.89±0.21CB</td>
</tr>
<tr>
<td>AlAsO$_4$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No plant</td>
<td>0.005±0.0A</td>
<td>8.02±0.38D</td>
</tr>
<tr>
<td><em>P. vittata</em></td>
<td>5.04±0.65C</td>
<td>7.37±1.64C, D</td>
</tr>
</tbody>
</table>

* The values are mean ± SE of three replicates. Same letters following means for As or DOC concentrations indicate no significant difference based on Tukey’s mean grouping at α=0.05. The two parameters measured in the two treatments have been considered as separate groups, i.e. As concentration and DOC concentration. The detection limit for arsenic was 5 µg L$^{-1}$. 


Table 4-2. Effect of arsenic-resistant bacteria (ARB) on As, Al and Fe concentrations (mg kg\(^{-1}\) As and Fe, and g kg\(^{-1}\) Al dw) in biomass of *P. vittata* after growing for 7 d in 0.25X Fe-free Hoagland medium, which was spiked with insoluble FeAsO\(_4\) and AlAsO\(_4\) minerals. Arsenic solubilized from root surface was obtained by washing in 1:1 HNO\(_3\). The values are mean±SE of three replicates. For statistical treatment, the means of arsenic concentration were analyzed together and Fe or Al concentrations were analyzed separately. Means followed by same letters were not significantly different based on Tukey’s mean grouping at α=0.05. Here the means have been separated in three groups, i.e. As concentration, Fe concentration and Al concentration.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tissue</th>
<th>As concentrations</th>
<th>Fe concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>With ARB</td>
<td>Control</td>
</tr>
<tr>
<td>FeAsO(_4)</td>
<td>Fronds</td>
<td>18.1±1.2A</td>
<td>35.3±2.6B</td>
</tr>
<tr>
<td></td>
<td>Roots-internal</td>
<td>19.4±1.8</td>
<td>450±27C</td>
</tr>
<tr>
<td></td>
<td>Roots-external</td>
<td>3.34±0.1D</td>
<td>36.3±1.26B</td>
</tr>
<tr>
<td></td>
<td>TF(^*)</td>
<td>0.9</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>Tissue</td>
<td>As concentrations</td>
<td>Al concentrations</td>
</tr>
<tr>
<td>AlAsO(_4)</td>
<td>Fronds</td>
<td>21.9±1.8</td>
<td>236±17E</td>
</tr>
<tr>
<td></td>
<td>Roots-internal</td>
<td>43.4±6.4B</td>
<td>180±12E</td>
</tr>
<tr>
<td></td>
<td>Roots-external</td>
<td>3.85±0.3D</td>
<td>9.99±0.4F</td>
</tr>
<tr>
<td></td>
<td>TF</td>
<td>0.5</td>
<td>1.3</td>
</tr>
</tbody>
</table>

\(^*\) TF= translocation factor, concentration ratio in the fronds to the roots
Table 4-3. Effect of arsenic-resistant bacteria (ARB) on biomass of *P. vittata* after growing for 7 d in 0.25X Fe-free Hoagland medium, which was spiked with insoluble FeAsO₄ and AlAsO₄ minerals. The values are mean ± SE of three replicates. Means followed by the same letters were not significantly different based on Tukey's mean grouping at α=0.05.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Biomass (g/plant dw)</th>
<th>Control</th>
<th>With ARB</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeAsO₄</td>
<td>fronds</td>
<td>6.10±0.29A</td>
<td>6.34±0.48A</td>
</tr>
<tr>
<td></td>
<td>roots</td>
<td>2.20±0.17C</td>
<td>4.16±0.24B</td>
</tr>
<tr>
<td>AIAsO₄</td>
<td>fronds</td>
<td>6.23±0.49A</td>
<td>5.23±0.54A,B</td>
</tr>
<tr>
<td></td>
<td>roots</td>
<td>1.51±0.36C</td>
<td>3.43±0.07B</td>
</tr>
</tbody>
</table>
Figure 4-1. Effects of ARB and *Bacillus* sp. on biomass (A) and P concentration in the biomass (B) of *P. vittata* (control with no bacteria added) after growing in 0.25X Fe-free Hoagland solution containing either FeAsO$_4$ or AlAsO$_4$ for 7 days. The values are mean ± SE of three replicates. Means indicated by the same letters were not significantly different based on Tukey’s mean grouping at $\alpha=0.05$. 
Phosphorus is a major nutrient essential for growth and development of all living organisms including plants. Total amount of phosphorus present in soil is around 400-1200 mg kg$^{-1}$ but most of it is insoluble. The concentration of soluble P in soil is only around 1 ppm. Phosphorus reserve in natural soil include both mineral and/or organic. Usually, agricultural soils accumulate large reserves of phosphorus from application of inorganic phosphorus fertilizers. In acidic soils phosphorus mostly gets immobilized by free iron and aluminum oxides and hydroxides while in alkaline soils it is immobilized by calcium. Contribution of organic phosphorus reserve in soil may range from 5% to as high as 95%. Inositol phosphate (soil phytate) constitutes around 50% of the total organic phosphorus in soil. Mostly soil microflora and plants store phosphorus as phytate, so it is the most insoluble and stable form of organic phosphorus (Rodriguez and Fraga 1999).

In soils, both plants and microorganisms can extract phosphorus from inorganic and organic reserves by the production of organic acids and phosphatase enzymes. Plant growth promoting rhizobacteria (PGPR) like *Pseudomonas, Bacillus, Burkholderia* etc. associated with the rhizosphere of the plants have the ability to solubilize phosphorus and make it more available to the plant (Rodriguez and Fraga, 1999). Most microorganisms secrete organic acids, which may be able to solubilize P from insoluble soil minerals. We found in our previous study that siderophores may be able to release As from insoluble soil minerals lie Fe/AlAsO$_4$. In this study we studied the solubilizing ability of siderophores in releasing P from insoluble soil minerals like FePO$_4$ and rock phosphate. Siderophores are low molecular weight organic ligands with high affinity and
specificity for iron binding. The ability to produce and assimilate siderophores with high affinities for FeIII gives a microorganism an important selective advantage under iron-limited conditions (Kraemer 2004).

Following the results from the experiment discussed in Chapter 4 we developed experiments to test the ability of siderophores produced by the ARB in solubilizing Fe and/or P from insoluble soil minerals like FePO₄ and rock phosphate. The ARB were isolated from the rhizosphere of _P. vittata_ and were exposed to sources of organic forms of P like phytate stored in plant biomass, which were deposited in the rhizosphere due to natural processes of plant decay.

The objectives of this research were to assess the ability of ARB in solubilizing P from inorganic minerals like FePO₄, phosphate rock and organic form like phytate. Further we tested if this phenomenon can enhance P and Fe uptake in the case of FePO₄ and only P in tomato plants and improve plant growth and nutrition.

**Materials and Methods**

**Solubilization of Rock Phosphate, FePO₄ and Phytate by Arsenic-Resistant Bacteria**

For the P solubilization experiments we used three insoluble P minerals: phosphate rock, FePO₄ and phytate. Phosphate Rock (Ca₁₀(PO₄)₆F₂) was a natural mineral collected from a mining site in Florida. FePO₄ was purchased as Iron (III) phosphate dihydrate (FePO₄·H₂O, Sigma-Aldrich). Phytate (empirical formula C₆H₁₈O₂₄P₆·xNa⁺·yH₂O) was also purchased as phytic acid sodium salt hydrate from rice (Sigma-Aldrich).

We used the spent modified LB growth medium of the 7 ARB strains and a control bacterium _Pseudomonas chlororaphis_ 63-28 (the same bacteria used in Chapter
3). The siderophores are extracellular secretions produced by bacteria, which are released into the spent growth medium. This medium containing the siderophores can solubilize insoluble P minerals. The 8 bacteria were grown in modified LB medium for 24 h at 30°C under 200 rpm shaking condition. Then half of the spent medium was filtered through 0.2 µM membrane filter to remove bacteria and half was boiled for 5 mins to reduce most of the enzyme activity. Preliminary we designed small scale experiments with 1 ml of spent medium which was mixed with 0.1 g of rock phosphate (PR), 0.01 g of FePO₄ or 0.04 g phytate (PA) and then this 1 ml mixture was incubated for 24 h at 30°C.

To test whether P was solubilized by siderophores via Fe chelation, we used wild type and two mutants of *Pseudomonas fluorescens* Pf-5 (the same strains were used in Chapter 4). In this experiment we used 1 ml of the filter-sterilized spent medium from the three strains and incubated for 24 h at 30°C with 0.01 g FePO₄. The water soluble P was measured by using modified molybdenum blue method.

**Arsenic solubilization by Arsenic-Resistant Bacteria PG12 and PG6 and impact on Tomato Growth**

Four bacterial cultures, PG6, 12, *P. chlororaphis* and PchA (*P. fluorescens* mutant) were grown in 75 ml liquid modified LB broth medium to an OD600 of 1.5 for 24 h at 30°C under 200 rpm shaking conditions. Then it was filtered through 0.2 µM membrane filter to remove bacteria, which was used for the following tomato experiment.

Tomato seedlings were grown in Fafard 2 germinating mix for around a month before being transferred to 0.2 strength Hoagland’s nutrient solution at pH 7. Three Hoagland’s solutions were used: 1) regular 0.2 strength Hoagland’s solution, 2) with the
P concentration at 1 mg/L and no chelated Fe; and 3) with the P concentration at 1 mg/L. The tomato seedlings were acclimated in these growth media for 35 d. The pH was checked regularly and the volume was maintained to 1 L in each aerated container.

For this experiment we selected four bacterial strains, PG12, *P. chlororaphis*, PG6 and PchA mutant of *P. fluorescens*. We used PG12 as our test strain in FePO₄ treatment because its spent growth medium solubilized the highest amount of P (9.5 mg L⁻¹) compared to our control bacteria PchA mutant, which solubilized extremely small amount of P from FePO₄. Similarly, we selected PG6 as our test strain for the phytate treatment as it solubilized most P (1.25 mg L⁻¹) from PA compared the control bacteria *P. chlororaphis*, which released insignificant amount of P from PA.

The tomato plants acclimated in low P + no Fe treatment received 20 ml spent medium from PG12 and *Pseudomonas fluorescens* Pf-5 (PchA) bacterial strains and 0.2 g of FePO₄ for Fe solubilization test by siderophores in the spent medium and tomato plants. The tomato plants acclimated in low P received 20 ml spent medium from PG6 and *Pseudomonas chlororaphis* 63-28 bacterial strains plus 0.2 g of phytate for P solubilization by P solubilizing enzymes in the spent medium and tomato plants. The plants grew for 1 week with 3 replicates. There were two additional controls: no FePO₄ was added to the low P+ no Fe spent medium of tomato plants or no PA was added to the low P spent growth medium of tomato plants. There was also a positive control, which was maintained in 0.2 strength Hoagland’s solution over the entire experiment period.

After 7-d of growth, plant biomass (dw) was determined after drying in an oven at 80°C for 2 d. To determine the potential role of P in enhancing plant growth, dried plant
samples were digested using a H₂SO₄/H₂O₂ to analyze P using modified molybdenum blue method. In addition to P, total Fe concentrations in the plants were measured in the same extracts. Fe concentrations in the supernatants and plant digest were determined using inductively-coupled plasma emission spectroscopy (PerkinElmer 5300DV, Waltham, MA) via EPA Method 2007.

Results and Discussion

Solubilization of Phosphate Rock, FePO₄ and Phytate

The bacterial strains PG5 and PG12, the two higher siderophore producing ARB (Table 2-3), were also most efficient in solubilizing P from PR i.e. 2.86 mg L⁻¹ (PG12) and 2.09 mg L⁻¹ (PG5) (Figure 5-1). The overall amount solubilized from PR was limited due to the low chelation capacity of siderophores with Ca ions (Kraemer 2004). This was the reason why PR was not included for further study. Similarly, PG12, which produced the highest amount of siderophores, solubilized the highest amount of P from FePO₄ (9.5 mg L⁻¹) (Figure 5-2).

The stability of Fe-siderophore complex is higher than that of Ca-siderophore. The 1:1 stability constants of Fe(III) siderophore complexes are between 10²³ and 10⁵² (Albrecht-Gary and Crumbliss 1998; Ams et al. 2002) compared to 10²⁰ for the iron(III)EDTA complex (Martell et al. 1995). For example, the Fe(III) complex of the trihydroxamate siderophore desferrioxamine-B (DFO-B) has a 1:1 stability constant of 10³⁰.⁶. This is many orders of magnitude higher than the corresponding Al(III) complex (K = 10²⁴.¹) or the Ca(II) complex (K = 10²⁶.⁴) (Kraemer et al. 2004). This is consistent with the low P solubilized by bacteria from PR. These results suggested that P was solubilized by the siderophores so we did a further experiment with wildtype and two
mutant bacterial strains of *Pseudomonas fluorescens* Pf-5, which were impaired in siderophore production.

The results from the P solubilization from FePO₄ by *Pseudomonas fluorescens* Pf-5 mutants show that the mutant PchA with complete impaired siderophore production solubilized significantly less amount of P compared to the mutant PchC (Hartney et al. 2011), which still produced some siderophores and the wildtype, which had the normal siderophore production. Hence, we can conclude that siderophores were probably responsible for releasing P from FePO₄. We also selected the mutant PchA as the control bacterium in our plant experiment due to its low P solubilization (1.1 mg L⁻¹) property compared to the wild type that solubilized 4.4 mg L⁻¹ P from FePO₄.

The results from the P solubilization from phytate demonstrated that all PG strains solubilized higher amount of P from PA compared to the control strain *P. chlororaphis* (Figure 5-3). Even though the overall solubilization rate was low but PG6 had the highest P solubilizing rate in 1 d, so we used this bacteria for the plant nutrition experiment along with the control bacteria *P. chlororaphis* with the least P solubilization.

Arsenate is a structural analog of phosphate, and is taken up into living cells by phosphate transporters. As(V) is insoluble in soils, as it reacts with calcium and iron, forming insoluble compounds (Silver and Phung 2005). Despite its well-established toxicity to life, arsenic resistance was discovered in microorganisms, which are able to survive normally in the presence of high concentrations of As(V). However, As(V) may compete with P ions for entering into the bacterial cell through P transporters. This phenomenon may stimulate P deficiency in the bacterial cell for more P sequestration even though adequate P may be present in the environment. These ARB may be more
competitive in solubilization of P from the insoluble forms of P in soil as well as in P uptake to survive under low P environment compared to the control strain *Pseudomonas chlororaphis* 63-28, which was isolated from the roots of canola (Paulitz et al. 2000). We also used another control bacterium PchC, which was a mutant of *Pseudomonas fluorescens* Pf-5, with impaired siderophore production. It solubilized 10 times less P from FePO₄ compared to our test strain PG12 (Figure 5-2).

**Plant Growth and Nutrient Uptake in Tomato**

The plant-bacteria experiment showed that the tomato plants benefitted from the solubilization of both P and Fe from FePO₄ and P from phytate. We also analyzed the Fe concentration in the controls and the FePO₄ treatments. The results showed that Fe concentrations increased around 10 times (10.8 g kg⁻¹ dw) in the shoots with PG12 treatment compared to PchA and control (Low P-Fe) and approximately double compared to the positive control (0.2 mg L⁻¹ Fe was present in the Hoagland nutrient solution). Thus the PG12 treatment not only improved plant growth by solubilizing P from FePO₄ but it also facilitated Fe uptake in tomato (Table 5-1).

Figure 5-5 shows the morphological changes in the tomato plants with the phytate treatment and Figure 5-6 shows the morphological changes in the tomato plants with the FePO₄ treatment. For the phytate +PG6 treatment, the dry weight of the shoot increased 65% (0.56 g dw⁻¹/plant) compared to the control (low P+Fe), i.e., 0.34 g dw⁻¹/plant and 44% more compared to *P. chlororaphis* (0.39 g dw⁻¹/plant) treatment (Figure 5-7A). The plant roots with PG6 treatment had (9.2 mg g⁻¹ dw) 2.2 times higher P concentration than that in the control, i.e., 4.13 mg g⁻¹ dw and 1.2 times higher than that in the *P. chlororaphis* treatment (7.57 mg g⁻¹ dw). The shoots with PG6 had 1.8 times higher P (8.63 mg g⁻¹ dw) compared to the control and the *P. chlororaphis* treatment.
(4.7 mg g\(^{-1}\) dw) (Figure 5-7B). The root dry weight (Figure 5-8A) after 7 d of FePO\(_4\) + PG12 treatment was 2.3 times (0.07 g dw\(^{-1}\)/plant) higher than that in the control (low P – Fe) and PchA treatment (0.03 g dw\(^{-1}\)/plant). The shoot biomass in the PG12 treatment was four times higher (0.04 g dw\(^{-1}\)/plant) than that in the control (0.01 g dw\(^{-1}\)/plant) and two times higher than that in PchA (0.02 g dw\(^{-1}\)/plant) treatment. We also measured the P concentration in the tomato plants and the roots with PG12 treatment had (11.6 mg g\(^{-1}\) dw) 2.7 times higher P concentration than that in the control, i.e., 4.27 mg g\(^{-1}\) dw and 1.7 times higher than that in the PchA treatment (7.0 mg g\(^{-1}\) dw). The shoots with PG12 had 1.5 times higher P (7.46 mg g\(^{-1}\) dw) compared to the control, i.e., 5.5 mg g\(^{-1}\) dw and 1.3 times higher than the PchA treatment (5.5 mg g\(^{-1}\) dw) (Figure 5-10). Plants support associated bacteria in their rhizosphere by exuding a significant portion of synthesized carbon. Some of these PGPR can influence plant growth by production of extracellular metabolites that can enhance plant growth (Sessitch and Puschenreiter 2008). Previous studies conducted in maize showed that seeds coated with P solubilizing bacteria increased grain yield by 64-85% compared to the seeds that were not coated with bacteria under field conditions due to solubilization of phosphate rock (Hameeda et al. 2008). Still the prospect of evaluating the role of siderophores in releasing insoluble plant nutrients complexed with Fe in soil have not been explored. Our study is the first one of its kind demonstrating the ability of PGPR from a metal tolerant plant can improve plant nutrition in a completely different food plant like tomato.

**Concluding Remarks**

From all the above data, we can conclude that the spent growth media from PG strains solubilized P from both organic (phytate) and inorganic (FePO\(_4\)) and improved plant growth and nutrition uptake. Siderophore-producing bacteria PG12 also made Fe
more available to the plant by increasing Fe uptake by tomato plants. These bacterial strains may have a potential to be used in field to improve plant nutrition.
Table 5-1. Fe concentration in the plant biomass of the control (full P+Fe), PchA (mutant impaired in siderophore production) and PG12 (arsenic-resistant bacterium) treatments in 0.2 strength Hoagland growth solution spiked with 0.2 g of FePO₄ for 7 d. The bars represent mean ± SE of three replicates.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tissue</th>
<th>Fe concentrations (g Kg⁻¹ dw)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FePO₄</td>
<td>Shoot</td>
<td>5.04±0.9A 1.3±0.0B 10.8±1.5C</td>
</tr>
<tr>
<td></td>
<td>Roots</td>
<td>225±11D 150±15E 270±6.8F</td>
</tr>
</tbody>
</table>

*Control here means plants grown in 0.2X Hoagland’s solution over the acclimation and treatment time.

**PG12 treatment means addition of 20 ml spent growth medium for each replicate.
Figure 5-1. P solubilization by the 7 ARB; PG4, 5, 6, 9, 10, 12, 16 and control strain *P. chlororaphis* in 1 ml spent modified LB medium (SGM) [in which the bacteria were grown for 24 h then filtered through 0.2 µM membrane filter], spiked with sterilized 0.1 g of phosphate rock (PR). The bars represent mean ± SE of three replicates.
Figure 5-2. P solubilization by the 7 ARB; PG4, 5, 6, 9, 10, 12, 16 and control strain *P. chlororaphis* in 1 ml spent modified LB medium (SGM) [in which the bacteria were grown for 24 h then filtered through 0.2 µM membrane filter], spiked with sterilized 0.01 g of FePO₄. The bars represent mean ± SE of three replicates.
Figure 5-3. P solubilization by the 7 ARB; PG4, 5, 6, 9, 10, 12 and 16 and control strain *P. chlororaphis* in 1 ml spent modified LB medium (SGM) [in which the bacteria were grown for 24 h then filtered through 0.2 µM membrane filter], spiked with sterilized 0.04 g of rice phytic acid. The bars represent mean ± SE of three replicates.
Figure 5-4. P solubilization by the wildtype and two mutants of *Pseudomonas fluorescens* Pf-5 in 1 ml spent modified LB medium (SGM) [in which the bacteria were grown for 24 h then filtered through 0.2 µM membrane filter], spiked with sterilized 0.01 g of FePO₄. The bars represent mean ± SE of three replicates.
Figure 5-5. Morphological changes in tomato plants in the control (low P+Fe) (A) and phytate treatments with spent growth medium of *Pseudomonas chlororaphis* (B) and PG6 (C) bacterial strains.
Figure 5-6. Morphological changes in tomato plants in the control (low P – Fe) (A) and phytate treatments with spent growth medium of PchA mutant (B), PG12 (C) bacterial strains and close up on PG12 treatment (D).
Figure 5-7. Rate of change of biomass (A) and change of P concentration in plant dry biomass (B) corresponding to the P solubilizing capabilities of the control (Low P+Fe), P.c. (*Pseudomonas chlororaphis*) and PG6 (test strain) in 0.2 strength Hoagland growth solution spiked with 0.2 g of phytate for 7 d. The bars represent mean ± SE of three replicates.
Figure 5-8. Change in tomato biomass (A) and change in P concentration in plant dry biomass (B) corresponding to the P and Fe solubilizing capabilities of the control (Low P-Fe), PchA (mutant impaired in siderophore production) and PG12 (test strain) in 0.2 strength Hoagland growth solution spiked with 0.2 g of FePO₄ for 7 d. The bars represent mean ± SE of three replicates.
CHAPTER 7
SUMMARY

The two elements, As and P have completely different roles in the environment. Arsenic is a lethal poison whereas P is the basis of life. However different the roles of these elements may be but these two chemical forms As(V) and P are structural analogs and chemically they behave similarly in the environment. Arsenate mimics P and is taken up into living cells through the same transporters. Our results clearly showed that P was preferentially taken into the living cells compared to As(V). So, the availability of P in the soil solution determines the rate of As(V) uptake into the bacterial cells.

Arsenic biogeochemical cycling in the environment is mainly controlled by microorganisms. The arsenic resistant-bacteria present in soil and spent media reduced As(V) to As(III), which is a more mobile form of As as it is soluble in water. Arsenite can be methylated into organic arsenic species, which are less harmful to living organisms. Arsenate reduction by bacteria can occur under both aerobic and anaerobic conditions. Our experimental conditions included only aerobic conditions as we were studying the rhizosphere of *P. vittata*. Aerobic As(V) reduction takes place via ars operon, which is activated by the entrance of As(V) in the cytosol. As(V) is reduced to As(III) in the cytosol and then it is expelled out of the cell. The entry of As(V) takes place via P transporters, which is controlled by the P concentration in the soil solution. Hence the availability of P in the soil controls the rate of As(V) reduction.

The product of As(V) reduction is As(III), which may also enter living cells via aquaporins. In soil the most abundant As species is As(V) so the rate of As(V) reduction will be proportional to the rate As(III) produced, which is also highly toxic like As(V).
Hence P availability also indirectly controls the entry of As(III) into the bacterial cells. Most bacteria studied so far are incapable of As(III) oxidation and expelling As(V) out of their cells. Most of the As(III) entering the bacterial cells is retained in the cell biomass and may get toxic for the cells after further accumulation. The ARB we isolated from the As-rich rhizosphere of *P. vittata* possessed the unique ability of performing both As(V) reduction and As(III) oxidation.

Arsenic-resistant bacteria that can expel both As(V) and As(III) out of their cells and may find it easier to survive under high As and low P aerobic soil environment like the rhizosphere of *P. vittata*. In this way the bacteria are able to remove both As(V) and As(III) out of their cells to retain less amount of As within the cells. This cyclical transformation between As(V) and As(III) may be beneficial adaptation by ARB surviving under high As conditions. The other half of As cycling by bacteria in soil, i.e. oxidation of As(III) produced by As(V) reduction has not been studied widely. Further research may add on to an interesting aspect in understanding the mechanism of As tolerance in soil bacteria.

The isolated ARB were fluorescent due to the production of water-soluble Fe-chelating compounds called siderophores. We investigated if these siderophores had a role in influencing the cycling of As species in the environment. Our results showed that siderophore-producing bacteria were more effective in oxidizing As(III) and that helped them being relatively more tolerant to As(III) compared to As(V). The ARB producing lower amount of siderophores were better As(V) reducers and hence more tolerant to As(V). Even though we found that mutants of *P. fluorescens* that were impaired in siderophore production had slower rates of As(III) oxidation and As(V) reduction, but we
could not find a direct relationship among Fe(III) uptake into the bacterial cells by siderophores and stimulation of As(III) oxidation. The idea of siderophore producing bacteria controlling As cycling by influencing As(V) reduction and As(III) oxidation needs further elucidation. The constitutional structure of the oxidase and reductase enzymes governing the biotransformation needs to be studied in detail to obtain further conclusions on the role of siderophores as drivers of As(III) oxidation.

The siderophores from ARB solubilized As and Fe/Al from FeAsO₄ and AlAsO₄ and also P and Fe from FePO₄. This was the first experimental study that demonstrated that siderophores produced by the rhizobacteria of *P. vittata* can use to benefit the growth of another plant like tomato. The ions were also made available to plants for uptake that benefitted their overall growth and increased As uptake by *P. vittata*. We also observed that both *P. vittata* and ARB increased the P uptake in the presence of As, due to As-induced P uptake. As the siderophores produced by ARB can solubilize both As and P, then this may help the arsenic-resistant plant and bacteria to thrive better under As-rich low P soil conditions. We also observed that the ARB showed less P uptake under the influence of As compared to our control strain isolated from regular soil. This difference may be due to the adaptation of ARB in dealing with As constantly in soil where P is extremely limited. Our results did not clearly explain the significance of this unusual property of ARB and this area requires extensive research to figure out the cause for stimulation of As-induced P uptake and mechanism behind its control.

We tried to study the P solubilizing ability of the ARB which may have to deal with increased P uptake demand in the As-rich rhizosphere of *P. vittata*. All the ARB were more effective in solubilizing P from both FePO₄ and phytate. Results from the
tomato experiment showed that the ARB enhanced both Fe and P uptake, which may stimulate plant growth by improving plant nutrition. According to our hypothesis, Fe will chelate with the bacterial siderophores and this would make Fe available to the bacteria for their Fe nutrition. Our results did not clearly demonstrate the mechanism by which the Fe from the Fe-siderophore complex was made available to plants. It is well known that Fe nutrition takes place in tomato by ferric reductase enzyme system but detailed information on Fe nutrition in ferns is not available. Better understanding of plant-bacteria interaction in rhizosphere on solubilization of Fe and uptake is necessary for using these fluorescent bacteria in improving Fe nutrition in food crops.

Further research in the areas discussed above would clear the ambiguity of concepts for practical application of As-resistant bacterial isolates for field application in improving Fe and P nutrition in tomato and *P. vittata*. The ARB also show promise in improving As uptake by *P. vittata* by solubilizing As from insoluble soil minerals.
Bacterial Growth medium

Modified LB medium
0.50% (wt/v) Tryptone
0.25% (wt/v) Yeast Extract
1.00% (wt/v) Sodium Chloride (NaCl)
2 mM Glucose
pH 7

Modified M9 medium for CAS assay

The medium for Chrome Azurol S (CAS) assay solution consisted of four different solutions that were mixed together.

The Fe-CAS indicator solution

10ml of 1 mM FeCl₃·6H₂O (in 10mM HCl) was mixed with 50 ml aqueous solution of CAS (1.21 mg ml⁻¹). Then this mixture was added slowly to 40 ml of an aqueous solution of HDTMA (1.82 mg ml⁻¹). The final solution was a dark blue solution which was autoclaved. This indicator solution was prepared freshly for each batch of CAS assay.

The buffer solution

9.76 g of MES was dissolved in 50ml of water. The pH was adjusted to 5.6 with 50% KOH, and the buffer solution was added to the dye solution to a volume of 100 ml.

The salt solution

This solution contained 0.3 g KH₂PO₄, 0.5 g NaCl, and 1.0 g NH₄Cl at pH 6.8 in 700 ml of water. The solution was autoclaved separately.
The micronutrients solution

2 g glucose, 2 g mannitol, 493 mg MgSO₄·7 H₂O, 11 mg CaCl₂, 1.17 mg MnSO₄·H₂O,
1.4 mg H₃BO₃, 0.04 mg CuSO₄·5H₂O, 1.2 mg ZnSO₄·7H₂O and 1.0 mg Na₂MoO₄·2H₂O
was added to 70 ml water. This solution was autoclaved before adding to the buffer
solution along with 30 ml 10% (w/v) casamino acids. This solution was filter-sterilized to
avoid precipitation. The indicator solution was added last with adequate stirring. The
modified M9 medium was used to grow the bacteria that had the same chemical
composition as CAS medium without the addition of the CAS dye.

**Hoagland’s plant growth medium**

Reagents and solutions (Gamborg and Water, 1975)

*Solution A:*

- 280 mg H₃BO₃
- 340 mg MnSO₄·H₂O
- 10 mg CuSO₄·5H₂O
- 22 mg ZnSO₄·7H₂O
- 10 mg (NH₄)₆Mo₇O₂₄·4H₂O

The volume adjusted to 100 ml with deionized H₂O

Stored at 4°C

*Solution B:*

- 0.5 ml concentrated H₂SO₄

The volume adjusted to 100 ml with deionized H₂O

Stored at 4°C

*Solution C:*

...
3.36 g Na₂EDTA
2.79 g FeSO₄
The volume adjusted to approximately 400 ml
Heat the solution to 70°C while stirring until the colour turns yellow-brown
Cooled down, the volume adjusted to 500 ml
Stored at 4°C

*Hoagland’s Nutrient Solution (1x):*

100 ml 10x stock solution
5 ml solution C
The volume adjusted to 1000 ml with deionized H₂O
Prepared just before use

*Hoagland’s Nutrient Solution (0.2x):*

20 ml 10x stock solution
1.0 ml solution C
The volume adjusted to 1000 ml with deionized H₂O
Prepared just before use.
APPENDIX B
ARSENIC SPECIATION IN THE GROWTH MEDIUM OF P. VITTA

The rhizobacteria from the roots of *P. vittata* transfers to the 0.2 strength Hoagland’s solution when grown in hydroponic set up. These bacteria control As speciation in the rhizosphere of *P. vittata*. The main objective of this study was to assess the effect of increasing dissolved organic carbon (DOC) on microbial redox balance in the spent Hoagland’s growth medium of *P. vittata* and *P. ensiformis*.

To prove this we grew *P. vittata* (PV) and As-sensitive control fern *P. ensiformis* (PE) for 7 days in 0.2 strength Hoagland’s solution. Then the spent growth solution from both these plants was divided into two sets, control and treatment. 1 ppm As(V) was added to both these sets. The treatment set received 25 µM of glucose and the control set was maintained with the original concentration of nutrients. These two experimental sets of spent growth solution were kept aerated for two days. Then the samples were analyzed for total As concentration and speciation by the procedure described in earlier chapters.

The results showed that there was no reduction of As(V) to As(III) in the case of both the plants, *P. vittata* and *P. ensiformis*. Addition of glucose or increase in DOC increased the rate of As(V) reduction by the bacteria present in the spent growth medium of the plants. There was 2.5% increase in As(V) reduction in the spent growth medium of *P. vittata* and 10% increase in As(V) reduction in *P. ensiformis* spent growth medium.

The research results discussed in the previous chapters prove that both As(V) reducing and As(III) oxidizing bacteria exist together in the rhizosphere in the *P.*
There is a dynamic equilibrium between As(V) reduction and As(III) oxidation processes and under our experimental conditions the As(III) oxidation is dominant so no As(V) reduction is observed. Hence, the addition of DOC may be favoring the As(V) reducing bacteria and we recorded a net increase in As(V) reduction vs. As(III) oxidation as both these processes may be taking place simultaneously.
Figure A-1. Change in arsenic speciation in 0.2 strength spent Hoagland’s growth solution of *P. vittata* (PV) and *P. ensiformis* (PE) spiked with 1 mg L$^{-1}$ for 2 d. Here As(T) represents total As concentration in the Hoagland’s solution. The bars represent mean ± SE of three replicates.
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Piyasa Ghosh, the second of the two siblings was born and brought up in India. She started painting when she was four and won several awards in art and cartooning competitions all through school. As a young kid she got training in Indian classical and can play a harmonium. During her childhood she spent a lot of time hiking in the forests on the mountain of Himalayas with her mother and grandfather. This ignited her interests towards wildlife conservation and she pursued her BS in Zoology (Honors) in Calcutta University, West Bengal, India. While pursuing her MS in an interdisciplinary Environmental Sciences program in Calcutta University she was inspired by her father to focus on chemistry as he himself was a biochemist. She obtained the opportunity to work on her MS thesis with Dr. A.K. Guha at the Department of Biological Chemistry, Indian Association for the Cultivation of Science, Kolkata, India. This experience enlightened her about the extent of heavy metal contamination in the environment and the need for remediation. Her MS research focused on mercury biosorption on Aspergillus versicolor biomass. After this she was privileged to work with Dr. L. Q. Ma at Soil and Water Sciences department, University of Florida and continue her PhD research on the role of bacteria in arsenic accumulation by Pteris vittata.