CHARACTERIZATION OF ARSENIC RESISTANT BACTERIAL COMMUNITIES IN THE RHIZOSPHERE OF AN ARSENIC HYPERACCUMULATOR *Pteris vittata* L.

By

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To my undergraduate university Nankai

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The arsenic (As) hyperaccumulator fern *Pteris vittata* L. produces large amounts of root exudates, which are hypothesized to solubilize arsenic and maintain a unique rhizosphere microbial community. A group of rhizosphere arsenic resistant bacteria were isolated and identified from two arsenic-contaminated sites where *P. vittata* growed. Twelve aerobic or facultative anaerobic bacterial isolates (*Naxibacter sp*. AH4, *Mesorhizobium sp.* AH5, *Methylobacterium sp*. AH6, *Enterobacter sp*. AH10, *Pseudomonas sp.* AH21, AH34, AH43, AH45, *Bacillus sp.* AH22, *Acinetobacter sp.* AH23 and *Caryophanon sp.* AH28) were resistant to 400 mM arsenic, the highest level of arsenic resistance reported to date. Two levels of arsenic detoxifications were proposed and studied. General resistance mechanisms were investigated by studying microbial growth characteristics under osmotic /oxidative stresses induced by sodium arsenate, sodium chloride, polyethylene glycol 6000 (PEG6000), or hydrogen peroxide. Arsenic specific resistant mechanisms were determined by identifying the two functional *arsC* or *arrA* genes based on PCR and Southern Hybridization method. Similar hydrogen peroxide inhibitions with broad-host pathogen *Salmonella typhimurium* were observed, and bacteria grew better

under osmotic stress generated by arsenic than sodium chloride or PEG, suggesting the existence of cross-stress tolerances in the isolates. While no *arsC* in bacterial isolates was detected to be similar with PAO1 *arsC*, *arrA* homologous sequences were cloned from some of the strains, indicating variations of both detoxification mechanisms and functional genes in different bacterial genera.

CHAPTER 1
INTRODUCTION

1.1 Environmental Sources of Arsenic

1.1.1 Arsenic in the Environment

Carcinogenic, mutagenic and teratogenic (Plant et al., 2003) arsenic (As) is a major constituent in more than 245 minerals and is ubiquous in the environment (Mandal and Suzuki, 2002). It is responsible for bladder, kidney, liver, lung, and skin cancers and is listed as a Class A human carcinogen by the USEPA (Chen et al., 2002). Both acute and chronic poisoning to humans has raised great concerns, especially in heavily contaminated areas such as Bangladesh and West Bengal, India. The serious health problems were described as "the greatest mass poisoning in human history" by World Health Organization (Vaughan, 2006).

The average concentration of arsenic in terrestrial environments is around 1.5 to 3 mg/kg. Arsenic in the environmental comes from natural and anthropogenic sources. Arsenic is present in reducing marine sediment, iron deposits, sedimentary iron ores and manganese nodules and is commonly associated with iron hydroxides and sulfides. Among the 245 minerals, approximately 60% are arsenates, 20% sulfides and sulfo-salts and the remaining 20% includes arsenides, arsenites, oxides, silicates and elemental arsenic (Ritchie, 1980).

The levels of soil arsenic range from 0.1 to 40 mg/kg in various countries. Anthropogenic sources generally exceed natural sources by 3 to 1in the environment. Arsenic can substitute for Si, Al or Fe in silicates minerals, therefore, contaminated soils usually have arsenic-rich parent materials (Fitz and Wenzel, 2002).The utilization of natural resources by humans releases arsenic into the air, water and soil. Arsenic may accumulate in soil through use of arsenical pesticides, application of fertilizers, dusts from burning of fossil fuels, and disposal of industrial and animal wastes. It has been estimated that there are 41% of the superfund sites in the USA are contaminated with arsenic (EPA, 1997), 1.4 million contaminated sites within the European

Community impacted by arsenic (ETCS, 1998), and more than 10,000 arsenic contaminated sites reported in Australia (Smith et al., 2002). These anthropogenic sources will adversely affect plants, animals and microorganisms.

The main arsenic producers were USA, Russia, France, Mexico, Germany, Peru, Namibia, Sweden, and China, and these countries accounted for about 90% of the world production (Mandal and Suzuki, 2002). In the past, about 80% of arsenic consumption was for agriculture uses such as insecticides and pesticides. The inorganic arsenicals, primarily, sodium arsenite, were widely used since 1890 as weed killers, particularly as non-selective soil sterilants (Vaughan, 2006). Two thousand and five hundred tons of H_3AsO_4 were used as desiccants on 1,222,000 acres (about 495,000 ha) of U.S. cotton in 1964 (Fordyce et al., 1995). Fluor- chrome-arsenic-phenol (FCAP), chromated copper arsenate (CCA) and ammonical copper arsenate (ACA) were used in 99% of the arsenical wood preservatives (Perker, 1981). Several arsenic compounds are currently used for feed additives, such as H_3AsO_4, 3-nitro-4-hydroxy phenylarsonic acid, 4-nitrophenylarsonic acid etc (Mandal and Suzuki, 2002).

1.1.2 Biogeochemistry of Arsenic

Changes in arsenic speciation occurs both abiotically and biotically, the latter was catalyzed by organisms.

Arsenite oxidation can be catalyzed by iron oxides, manganese oxides and organic compounds when the oxidation potential is high enough and usually at low pH (< 3), though it is slow. Most arsenite is oxidized microbiologically as a detoxification mechanisms or as elector donor, which are known as heterotrophic arsenite oxidizers (HAOs) or chemolithoautotrophic arsenite oxidizers (CAOs) (Oremland and Stolz, 2003). HAOs incorporate a periplasmic enzyme to catalyze the oxidation reaction, which converts arsenite encountered on the cell's outer membrane. This presumably makes it less likely to enter the cell. On the other hand, CAOs use

arsenite as elector donor, reducing either oxygen or nitrate to obtain the energy generated in this process to fix CO_2.

While arsenate reduction at low pH occurres abiotically and is coupled with sulfide (HS^- or H_2S) oxidation, its reduction in neutral environments are mostly catalyzed by microorganisms, for either energy production or arsenic detoxification.

Referred to as dissimilatory arsenate-reducing prokaryotes (DARPs) (Plant et al., 2003), bacteria incorporating arsenate reduction in respiration usually growing in anaerobic environments and use arsenate as elector acceptor. Therefore, they are found to be able to grow in both oxic and anoxic conditions, where hydrogen as well as a variety of organic carbon sources including acetate, formate, pyruvate, butyrate, citrate, succinate, fumarate, malate, and glucose can be their electron donors (Dowdle et al., 1996).

While the respiratory arsenate reductases remains to be fully elucidated, the second arsenate reduction-detoxification system, known as an "*ars* operon", is found in many microorganisms and well understood both functionally and structurally. Since the arsenate/ arsenite oxidation/reduction potential is +135mV (Niggemyer et al., 2001), this type of arsenate reduction needs glutaredoxin, thioredoxin or ferredoxin as cofactor to reduce reaction potential.

Although the two resistant mechanisms function differently, the ability to respire arsenate does not preclude the presence of ars operon system. Recently, *Shewanella sp.* ANA-3 was found to have both respiratory and detoxifying arsenate reductases (Fitz et al., 2003).

1.2 Arsenic in Plants

1.2.1 Plant Arsenic Metabolisms

Most plants do not take up much arsenic, with average concentrations in plants being <3.6 mg/kg (Kabata-Pendias and Pendias, 2000). This is not only because arsenic concentration is low

and it is highly toxic to organisms, but also, like phosphorus, majority of arsenic in soils is insoluble and thus has low availability to plants (Tu et al., 2004).

Most studies on arsenic in plants concentrate on the transformation of arsenical pesticides in crops such as rice, tomato, apple or carrot. All plants growing on both arsenic-contaminated and uncontaminated sites have more than one arsenic species in their tissues. A range of arsenic compounds are found in plant tissues, for example, inorganic arsenite and arsenate, methylated arsenic species, arsenobetaine and arseno-sugars. Plant species, which are not resistant to arsenic suffer considerable stress upon exposure, with symptoms ranging from inhibition of root growth to death (Meharg and Hartley-Whitaker, 2002).

Inorganic arsenic species are generally highly toxic to plants. Mechanisms of arsenic uptake by plant roots are not clearly understood. It may occur either through uptake by phosphate transporters in mycorrhizal fungus (Sharples et al., 2000) or directly uptake by plant roots (Abedin et al., 2002). Arsenate acts as a phosphate analogue and is transported across the plasma membrane via phosphate transport systems. It competes with phosphate in all biomolecules. Arsenate can be reduced to arsenite non-enzymatically by glutaredoxin or enzymatically by specific arsenate reductase. Arsenite is taken up by the aquaglyceroporin.

Following the reduction of arsenate to arsenite in plants, arsenic may be potentially further metabolized to methylated species. Organic arsenic such as MMA, DMA, tetramethylarsonium ions (TETRA), trimethylarsonium oxide (TMAO), as well as arsenobetaine and arseno-sugars are found in plants; however, methylated arsenic species are present as a minor fraction of the arsenic burden in plants. It has not been proven whether these compounds are actually metabolized by the plant or simply taken up in those forms from soil solution. Organic arsenic species are generally considered to be less toxic than inorganic species to organisms; however, it

is suggested that they were more toxic than inorganic arsenic species in terrestrial plants (Meharg and Hartley-Whitaker, 2002).

Exposure to inorganic arsenic species results in the generation of reactive oxygen species (ROS), which probably occurs through the conversion of arsenate to arsenite and leads to synthesis of enzymatic antioxidants such as superoxide dismutase, catalase and glutathione-S-transferase, and nonenzymatic antioxidants like glutathione and ascorbate (Dat et al., 2000). Moreover, methylation is also thought to be redox driven and such reactions could give rise to ROS.

1.2.2 Arsenic Resistance Mechanisms in Plants

As an analogue of the macronutrient phosphorus, arsenic is somewhat unusual comparing with transition metals and metalloids. Plants growing on arsenate contaminated soils will assimilate high levels of arsenate unless they have altered phosphate transport mechanisms (Sharples et al., 2000). In spite of that, arsenate resistance has been identified in a number of plant species growing on arsenic contaminated soils including *Andropogon scoparius, Agrostis castellana, A. delicatula, A. capillaris, Deschampsia cespitosa,* and *Plantago lanceolata* (Meharg and Hartley-Whitaker, 2002). In those plants, resistance is generally achieved via suppression of the high affinity phosphate uptake system. It is thought that this suppression reduces arsenate influx to a level at which the plant can detoxify by constitutive mechanisms (Meharg and Macnair, 1992). Thus, arsenate sensitivity is intimately linked to phosphate nutrition, with increased phosphate status leading to reduced arsenate uptake (Meharg et al., 1994). Indeed, most arsenate resistant plants always suppress the high affinity uptake system and are insensitive to plant phosphorous status (Meharg and Macnair, 1992).

However, arsenic resistant plants can still accumulate considerable levels of arsenic in their tissues. Therefore it is assumed that arsenic resistant plants either compartmentalize and/or

transform arsenic to less phytotoxic arsenic species to withstand high cellular arsenic burdens, such as complexing with phytochelatins (PCs) (Meharg et al., 1994). PCs are synthesized from reduced glutathione (GSH) by the transpeptidation of γ-glutamylcysteinyl dipeptides, through the action of the constitutive enzyme PC synthase (Vatamaniuk et al., 2000). Synthesis of PCs is induced by a range of cations such as Ag^{+}, Cd^{2+}, Cu^{2+}, Hg^{2+} and Pb^{2+} and the oxyanions arsenate and selenate (Grill, 1987). Arsenite in many arsenic-resistant plant tissues is complexed with phytochelatins (PCs). For example, X-ray absorption spectroscopy (XAS) of *Brassica juncea* has determined that arsenic, when present as arsenite, is coordinated with three sulphur groups (Pickering et al., 2000). Meharg and Hartley-Whitaker (2002) showed that PCs are induced upon exposure to inorganic arsenic in cell cultures, root cultures, and enzyme preparations of different plants

In addition to glutathione's role as the precursor of PCs, it is also an antioxidant. Synthesis of PCs can therefore result in glutathione depletion, reducing the amount of antioxidant available for quenching ROS (Hartley-Whitaker et al., 2001).

Microorganisms associated with plants are also involved in heavy metal resistance. Free living and symbiotic bacteria and arbuscular mycorrhizal fungi play important roles in increasing nutrient storages, amplifying plant resistance to drought, enhancing plant salinity tolerance and cold hardiness, and strengthening plant resistance to heavy metals (Gyaneshwar et al., 2002; Lucy et al., 2004).

1.2. 3 Arsenic hyperaccumulator *Pteris Vittata* L.

Chinese brake fern (*Pteris Vittata* L.) is the first reported arsenic hyperaccumulator. It tolerates soil arsenic concentration up to 1500 mg/kg and rapidly accumulate up to 2.3% in its aboveground biomass from both uncontaminated and contaminated soil (Ma et al., 2001). Most

of the accumulated arsenic is concentrated in the epidermal layers of the fronds (Lombi et al., 2002) and is probably stored in the vacuoles (Pickering et al., 2006). In addition to the remarkable ability of *P. vittata* to tolerate high internal arsenic, its extraction of low levels of arsenate from soil is extraordinary, considering that arsenate mobility in soil is limited.

On one hand, arsenic detoxification and accumulation mechanisms by *P. vittata* have been investigated, but it is still not fully elucidated. *P. vittata* can hyperaccumulate arsenite and MMA, suggesting that alteration of phosphate transporter may not be involved in hyperaccumulation (Meharg and Hartley-Whitaker, 2002). On the other hand, explanation from evolutionary aspect has been proposed. Hyperaccumulation may serve as a means of avoiding competition or a defense strategy against herbivores and pathogens to gain ecological advantage, since most plants are sensitive to arsenic. Hyperaccumulator turns out to be toxic for herbivores such as grass hoppers (Rathinasabapathi et al., 2007). Arsenic hyperaccumulation by *P. vittata* may be a strategy for attaining metal resistance by accumulation and sequestration, or may be a result of inadvertent uptake of arsenate, which enhances its ability in phosphate acquisition (Rathinasabapathi, 2006).

1.3 Microbial Arsenic Resistance

1.3.1 Arsenic Resistant Bacteria

Bacteria living under environmental stresses have evolved different systems to withstand the growth restriction, where a number of genes are activated or repressed to adapt cell physiology or metabolism to the environment. Those genes include both global regulators and some specific functional genes. The global regulators regulate a large number of genes and result in a very different transcriptional profile, while the specific functional genes encode specific enzymes responsible for counteracting environmental factors.

Arsenic detoxification is one of those stress tolerance mechanisms in bacteria. Although its concentration in crust is about 1 mg/kg, arsenic is widely distributed in natural environments and commonly associated with mineral ores like Cu, Au, Ag, Pb, and Sn, either as part of the mineral structure or as sorbed species (Smedley and Kinniburgh, 2002). It is nontoxic in the insoluble forms before they are chemically /biologically mobilized, which would produce high concentration of inorganic arsenite and/or arsenate, especially in some acid mine drainage. On the other hand, biological metabolism converts inorganic arsenic into monomethylated, dimethylated, and trimethylated organic arsenic species, which are more toxic than inorganic arsenic without further sequestration, though methylation and sequestration together is considered as one of the arsenic detoxification strategies (Stolz et al., 2006; Turpeinen et al., 1999). Therefore, bacteria play an important role in arsenic biogeochemistry, involving in biological reduction/oxidation, methylation/demethylation, precipitation/dissolution, and sorption/desorption. In consequence, different detoxifications strategies are developed to withstand the growth restriction when they expose to arsenic.

It is hypothesized that similar to bacterial detoxification principles under all environmental stresses; arsenic resistance systems can be categorized into two classes, general and specific systems. While the general systems alleviate arsenic induced cell toxicities such as oxidative burst or osmotic stress damage, the specific systems involve in arsenic transformation and sequestration. The two systems together accomplish arsenic detoxification at different cellular metabolic levels.

1. 3.2 General Arsenic Resistant Mechanisms

The first class of resistant mechanisms comprises more general stress related to gene regulations, which prepare bacteria to survive under different environmental stresses. These systems are generally turned on under hyper-osmotic condition or low nutrient environment, and

cross protect from other stresses such as oxidative burst, heavy metal stress and sodium hypochlorite (Pichereau et al., 2000).

In fact, multiple types of tolerance occur frequently in bacteria living under osmotic stress or starvation resulting from global reprogramming of gene expressions. Those results appeared in early literature. For example, Kjelleberg (1993) showed, upon stress, a rapid change in gene expression pattern in non-differentiating bacteria by a two-dimensional electrophoresis. Hartke (1998) showed that, induced under complete starvation condition in tap water, *Enterococcus faecalis* cells become more tolerating to heat, acid and sodium hypochlorite stresses, and was significantly more resistant to UV^{245} irradiation. Although the functional genes and physiological pathways were of fundamental interests to understand the general stress resistant mechanisms, it seemed impossible at that time to study at the transcriptional or post-transcriptional level because of technique and information limitations. However, as more new technologies being developed, scientists are now able to look into gene up or down regulation in more specific details. These techniques include microarray, quantitative real-time PCR or Northern blot in analyzing transcriptional profiles and more recent discovery, RNA in posttranscriptional regulation. For example, a recent study of gene profile changes responding to variation of pH in *Shigella flexneri* by whole-genome microarrays differentiated the expression of 307 genes, including global regulators such as the sigma factors and specific pH dependent and energy metabolic genes that increase acid production and energy generation (Cheng et al., 2007). Northern blot comparison of *Listeria monocytogenes* mRNA between growth in neutral and alkali environments revealed that expression of about 60 σ^B - regulated genes was significantly increased, and bacterial strain was more resistant to subsequent alkaline, osmotic or ethanol stress (Giotis et al., 2008). Those

researches discern that global regulons confer cross-protection against multiple stresses such as oxidative burst, osmotic stress, as well as heavy metal toxicities.

Bacterial growth under oxidative burst. An example of global regulators in bacteria is *oxyR* system, which is a regulon induced typically by hydrogen peroxide and tightly controls hydrogen peroxide level by increasing scavenging activities and limiting hydrogen peroxide generation in the respiratory chain. The connection between oxidative stress and arsenic detoxification is supported not only by evolutional view, but also by experimental results.

On one hand, the earlier age of life was in anaerobic environment; therefore, all fundamental enzymes were integrated into metabolisms in the absence of selective oxidative pressure, including As detoxification system. But after photosystem II appeared, microorganisms acquired oxygen tolerance and developed mechanisms to defend themselves against both As induced oxidative stress and superoxide (O^{2-}) which are inadvertently generated by-products of aerobic metabolisms (Imlay, 2008). OxyR is one of the functional systems prevailing among bacteria. Although it has been known for a long time, its regulation profile isn't clear until microarray technique was employed in recent studies. For example, Zheng et al. (2001) showed that the regulon was activated by a submicromolar of hydrogen peroxide and up or down regulated a large amount of genes, including 30 genes with >10-fold induction

On the other hand, arsenic detoxification is also found to be evolved in the early age of life, which is verified by phylogenetic analysis of arsenic resistant genes in both archaea and bacteria (Gihring et al., 2003; Jackson and Dugas, 2003). These studies showed arsenic detoxification results from either variation in *ars* operon genes or phylogenetical separation of bacterial, archaeal, and eukaryotic in the same gene in the operon. Therefore, at early age, metabolisms of

microorganisms living in environments with geological source of arsenic are under influence of both arsenic and superoxide, which lead to overlap in bacterial metabolisms.

Arsenic has been shown to mediate its toxicity through induced generation of reactive oxygen species. Therefore, it is not a surprise to see the correlation between their arsenic resistance and anti- hydrogen peroxide ability (Gihring et al., 2003; Liu et al., 2001). Moreover, previous study showed that *oxyR* mutation increased sensitivity to both arsenate and arsenite in a plant pathogenic bacterium, *Xanthomonas campestris* pv. phaseoli (*Xp*) (Sukchawalit et al., 2005). It is reasonable to assume that, while specific arsenic detoxification systems directly eliminate toxic arsenic, other oxidative response systems such as OxyR scavenge downstream oxidative burst toxicity induced by arsenic, and thus reduce toxicities at different levels.

Growth under osmotic stress. Many studies on heavy metal resistance and osmotic stress in bacteria suggested that, bacteria are capable of many chemical transformations of heavy metals, including oxidation, reduction, methylation, demethylation, complexation, and precipitation. However, these transformations are sometimes byproducts of normal metabolism (Silver and Misra, 1984), therefore making the effort to search for functional genes became difficult. From this angle, cell structural or physiological specialties are also important in studying general resistant mechanisms. For example, detoxification via immobilization can result from sorption to biomass or exopolymers, sequestration, crystallization or precipitation as organic and inorganic compounds intracellularly. Reduction/oxidation of different species may result in immobilization, e.g., MnII to MnIV, CrVI to CrIII, and arsenite to arsenate. Those transformation are not only affected by bacterial functional enzymes, but also the reduction/oxidation potential in the environment, which are greatly influenced by inhabited microbial communities (Gadd, 2004).

Deposition of metals onto bacterial cell wall/membrane carboxylic, amino, thiol, hydroxyl, and hydro-carboxylic functional groups are well-known phenomenon. Although there is no direct evidence, a previous study showed arsenate may interfere with the regulation of cell wall biosynthesis, resulting from arsenic binding onto cell walls (Mandal et al., 2008). Moreover, data analysis revealed similar functional heavy metal-binding peptides phytochelatins (PCs) in prokaryotes, such as *cyanobacteria nostoc (Anabaena)* sp. PCC 7120, *Prochlorococcus marinus* str. MIT9313 (BX572098), and *Anabaena variabilis* ATCC29413 (Hirata et al., 2005). Like heavy metal hyperaccumulation plants, PCs in bacteria would confer dramatically heavy metal tolerance.

1.3.3 Specific Arsenic Resistant Mechanisms

The second class of arsenic detoxification is via specific resistant system, which is induced by sublethal doses of arsenic, and permits survival against a challenging arsenic dose. Most arsenite is oxidized microbiologically, either by HAOs or CAOs (Oremland and Stolz, 2003). In terms of arsenate-reduction based mechanisms, so far two mechanisms have been reported, the DARPs and *ars* operon system.

DARPs usually grow in anaerobic environments using arsenate as elector acceptor to incorporate arsenate reduction in respiration. This happens when redox potential is below +135mV (arsenate/arsenite), allowing the reaction happen easily with the help of glutaredoxin, thioredoxin or ferredoxin (Plant et al., 2003). However, no “obligate” DARPs have been found, because all the strains examined can use other electron acceptors for growth, such as sulphate, phosphate, nitrate, MnIV and FeIII (Dowdle et al., 1996). For example, while nitrate was found to be the preferred electron acceptor and inhibited arsenate reduction by Dowdle et al. (1996), a laboratory cultured strain *Desulfomicrobium sp.* Ben-RB was found to reduce arsenate and sulfate at the same time using lactate as electron donor, where same sulfate concentration did not

inhibit arsenate reduction (Dowdle et al., 1996; Macy et al., 2000). Certain DARP species are more sensitive to arsenic than others, for example, haloalkaliphile *Bacillus selenitireducens* grows well at 10 mM arsenate, while *Sulfurospirillum* species is only able to grow at 5 mM. This is possibly because the product arsenite is charged at high pH and cannot exit the cell in alkaline condition where the latter one habited (Ahmann et al., 1994; Switzer Blum et al., 1998), and may be also due to the strong general resistant cell metabolisms.

Structural and functional study of arsenate reductase was first carried out in *Chrysiogenes arsenatis* (Gram negative) and *Bacillus selenitireducens* (Gram positive), both of which are encoded by typical *arr* operon. The protein is a heterodimer consisting of 87 kD (arrA) and 29 kD (arrB) subunits, both contain an iron sulfur cluster, placing it in the dimethylsulfoxide (DMSO) reductase family of mononuclear molybdenum enzyme (Krafft and Macy, 1998).

While the respiratory arsenate reductase remains to be fully elucidated, the second arsenate reduction-detoxification system, known as *ars* operon, is found in many microorganisms and well understood both functionally and structurally. Most heterotrophic bacteria have the chromosome *ars* operon with one set of genes of multiple set of genes. The operon in *Escherichia coli* SG20136 has both plasmid and chromosomal loci. The plasmid R733 consists of *arsA, arsB, arsC, arsD,* and *arsR*, whereas the chromosome has only *arsB, arsC,* and *arsR* (Rosen et al., 1988). Arsenate is bond by a cysteine residue near the N-terminal of ArsC, and is reduced with electrons donated by GSH (Rosen, 2002). Although arsenite is more toxic than the oxidized form arsenate, it can be excreted by an arsenite-specific transporter, *ArsB*, whose function depends on an ATPase arsA in *E. coli*. On the other hand, the *ars* operon in plasmid pI258 of *Staphylococcus aureus* consists of only *arsB, arsC,* and *arsD*, and the electron donor is reduced thioredoxin (Liu et al., 2004; Newman et al., 1997). Although the ATPase gene *arsA* is

absent in the operon, arsenite is expelled from the cell via an ATPase independent ArsB. ArsR and arsD are arsenite responsive repressors of the *ars* operon. In contrast to the DARPs, this system doesn't gain energy in the reduction reaction and occurs under oxic and anoxic condition (Macur et al., 2001b).

Though typical *ars* operon is comprised of the three-gene structure *arsRBC*, previous study showed a variation in *ars* operon in different bacterial genera. For example, while *E. coli* SG20136 has both plasmid chromosome loci, two complete *ars* operons (*ars1* and *ars2*) encoding *arsRBC,* and two orphan genes (*arsB3* and *arsC4*) are found in *Corynebacterium glutamicum* genome (Mateos et al., 2006). *Ochrobactrum tritici* SCII24^T also has two *ars* operons (named as *ars1* and *ars2*) encoding arsenic and antimony resistant genes, *ars1* contains five genes encoding arsR, arsD, arsA, CBS-domain-containing protein and arsB, and *ars2* encodes two arsR, two arsC, one ACR3 and one arsH-like protein (Branco et al., 2008). Another study of a large linear plasmid pHZ227 in *Streptomyces sp.* found two novel genes, *arsO* and *arsT*, which were coactivated and cotranscribed with *arsR, arsB* and *arsC* (Wang et al., 2006). ArsO is a flavin-binding monooxygenase, and arsT is a thioredoxin reductase.

The sequence, structure and functional analysis showed difference of functional genes among the two arsenate reductases in DARPs or *ars* operon. In addition, two unrelated clades of *arsC* sequences are found among those *ars* operon reported. Though their biochemical function is the same, there is no evolutionary relationship (Mukhopadhyay et al., 2002). The representatives of the two enzymes are *E. coli* SG20136 plasmid R773 arsC (Rosen, 2002) and *S. aureus* plasmid pI258 arsC (Mukhopadhyay et al., 2002). Both ArsC arsenate reductases are small monomeric protein of about 135 amino acid residues containing three essential cysteine residues, which are involved in a cascade sequence of enzyme activity. They use different active

cysteine sites and different cofactors, glutaredoxin and thioredoxin respectively, to reduce the reduction potential.

The enzyme arsC catalyzing arsenate reduction is located in the cytoplasm and is therefore only able to reduce arsenate that already entered the cell. This reduction-detoxification mechanism has been found in aerobic bacteria isolated from arsenic-contaminated soils and mine tailings (Jones et al., 2000; Macur et al., 2001a), which indicates the importance of this mechanism in the biogeochemical cycling of arsenic in nature (Inskeep et al., 2002).

1.4 Arsenic Hyperaccumulator *Pteris vitttata* L. as a Unique Model

Concentration of soluble phosphorus in soil is usually very low, normally at levels of 1 mg/kg or less (Belton et al., 1985). Arsenic concentrations are even lower in uncontaminated soils. However, the arsenic hyperaccumulator *P. vittata* is found to retrieve more bioavailable arsenic from recalcitrant pool (Fayiga et al., 2007). Understanding the interactions between roots, rhizosphere microbial communities, as well as rhizosphere dynamics of arsenic and phosphorus are important for improving phosphorus nutrition and reducing arsenic uptake in food and feed crops, and increase arsenic uptake in the hyperaccumulation plant.

The rhizosphere of *P. vittata* serves as a unique model for such investigations because of the unusual arsenic resistance and hyperaccumulation traits of the plant. The fact that the soil where arsenic hyperaccumulation is first found in *P. vittata* has been contaminated for about 60 years (Komar, 1999), coupled with the ability of *P. vittata* to solubilize arsenic and/or phosphorus in the rhizosphere, provides a great opportunity to discover microbes that may have developed new and/or unique tolerance mechanisms in arsenic resistance in the rhizosphere. Besides, it is reasonable that such high ability in accumulating arsenic from soil may partially result from the high ability of rhizosphere bacteria associated with the plant in mobilizing and tolerating arsenic.

Rhizosphere microbial communities are contributing to the biodegradation of old fronds fallen back to soil. While live fronds accumulate about 4000 mg/kg of arsenic, fronds undergoing the process of senescence have only 200-300 mg/kg of arsenic. The exploitation of the functional microbial communities involved in the process will provide potentially environmental friendly method for identifying arsenic-resistant bacteria and the associated mechanisms.

Fitz *et al*. (2002) investigated the changes in the rhizosphere characteristics of *P*. *vittata* relevant to its use in phytoextraction. They reported that arsenic was mainly acquired from less available pools. Although no information is available on the fate of As in the rhizosphere, it is known that certain environmental microorganisms have adapted to a variety of habitats using arsenic oxyanions for energy generation, either by oxidizing arsenite or by respiring arsenate (Lasat, 2002; Raghu and MacRae, 1966), thus catalyzing the arsenic biogeo-cycle in the environment. Soil microorganisms have an important role in mobilizing nutrients in the soil from recalcitrant sources. This is of great importance for phytoextraction of toxic metals because the heavy metal ions are made more available for root uptake by a more active rhizosphere microbial community (Schuster et al., 2003).

1.3.1 Arsenic-resistant microbial communities

The rhizosphere is the area where the roots are interacting with the neighboring plant species for space, water, and mineral nutrients, and with soil-borne microorganisms, including bacteria and fungi feeding on an abundant source of organic material (Ryan et al., 2001). Rhizosphere microbial communities are usually in contact with the root surface, or rhizoplane, providing available P, N and mineral nutrients to host plants.

While exposed to more bioavailable arsenic, rhizosphere microorganisms in *P. vittata* have to tolerate higher arsenic concentration. Microbial detoxification based on arsenate reduction has been well studied in *E. coli*, and has also been documented for *Staphylococcus, Bacillus,*

Acidithiobacillus, Pseudomonas, *Shewanella* and a large group of bacteria (Mateos et al., 2006). Scientists have successfully isolated high arsenic resistant bacteria and fungi from arsenic contaminated environment. For example, Anderson and Cook (2004) isolated a number of arsenic resistant chemoheterotrophic bacteria from two arsenic contaminated soils in New Zealand, which tolerated up to 7,500 mg/kg arsenate. Rathinasabapathi et al. (2006) isolated bacteria from the phyllosphere of *P. vittata*, which exhibited resistance to arsenate, arsenite, and antimony in the culture medium. Cánovas et al. (2003) isolated a filamentous fungus (*Aspergillus* sp. P37) from arsenic contaminated river in Spain, which is able to grow at 15,000 mg/kg arsenic. Among those documented arsenic resistant bacteria, *Corynebacterium glutamicum* is the most prominent, which tolerated up to 30,000 mg/kg of arsenate (Mateos et al., 2006).

Most of those studies have focused on the specific arsenic resistant mechanisms, i.e., functional genes in detoxification mechanisms through transformation of arsenic species and sequestration in either vacuoles or outer membrane; However, little information is available at global metabolic levels such as the osmotic stress when arsenic is stored/accumulated in outer membrane, the oxidative stress generated during arsenic exposure, and the impacts of those stresses on cellular growth.

1.3.2 Phosphorus solubilizing bacteria

There are considerable populations of phosphate-solubilizing microorganisms (PSMs) in soil, especially in the rhizosphere. These include both aerobic and anaerobic strains, with a prevalence of aerobic strains in submerged soils. PSMs render insoluble phosphate into soluble form via acidification, chelation and exchange reactions. This process not only reduces the cost of manufacturing fertilizers in industry but also mobilizes the fertilizers added to soil (Rodriguez and Reynaldo, 1999). Therefore, many researchers have tried to increase the plant-available

phosphate fraction using PSMs such as *Pseudomonas* (Suh et al., 1995), *Bacillus* (Raj et al., 1981), *Enterobacter* (Laheurte and Berthelin, 1988), *Agrobacterium* and *Aspergillus* (Varsha and Patel, 2000; Son et al., 2006).

Bacterial genera in this capacity include *Pseudomonas, Bacillus, Rhizobium, Burkholderia, Achromobacter, Agrobacterium, Micrococcus, Aereobacter, Flavobacterium a*nd *Erwinia.* Species from the genera *Pseudomonas, Bacillus and Rhizobium* are among the most efficient phosphate solubilizers. The presence of these bacteria is essential in providing available phosphate to plants (Hilda et al., 1999). A previous study has shown more root exudates in *P. vittata* than non-hyperaccumulator *Nephrolepis exaltata*, consisting mainly of phytic and oxalic acid (Tu et al., 2004). *P. vittata* takes up more phosphorus when exposed to arsenic, probably resulting from arsenic-induced phosphorus deficiency (Tu and Ma, 2003). As a nutrient, phytic acid sustains the organic phosphorus solubilizing microbial community. It is hypothesized that both organic and inorganic PSMs are more active in the rhizosphere of *P. vittata*, and therefore, increasing phosphorus and/or arsenic availability and possibly alleviating arsenic toxicity to the plant. However, there is no study conducted to understand the roles of those bacteria in the rhizosphere of *P. vittata*.

1.3.3 Arsenic resistant bacteria

Many engineering technologies have been developed for remediation of arsenic contaminated soil and water. Remediating contaminated water to achieve US Environmental Protection Agency's current standard of 0.01 mg /L arsenic can be achieved using technologies such as precipitation/coprecipitation, membrane filtration, adsorption, ion exchange and permeable reactive barriers. However, these technologies are expensive and time-consuming, and therefore, are not widely applicable. Phytoextraction, the use of green plants to clean up contaminated soil, has attracted attention as an environmentally friendly and low-input

remediation technique (Fitz and Wenzel, 2002). This technology makes use of hyperaccumulator plants that extract pollutants from the soil and accumulate them in the harvestable above-ground biomass. *P. vittata* offers a promising resource for phytoremediation, however, the technology is still in the development and more research is needed.

The rate of metal removal is the key to the success of phytoremediation, which depends upon both the plant biomass harvested and metal concentration in harvested biomass. Interactions between arsenic and phosphorus influence their availability in soils, and thus plant growth and uptake of arsenic and phosphorus. Quantitative analysis of kinetic parameters showed that phosphate inhibited arsenate influx in a directly competitive manner; consistent with the hypothesis that arsenate enters plant roots via phosphate transporters (Wang et al., 2002). However, addition of phosphate substantially increased plant biomass by alleviating arsenate phytotoxicity at high arsenate levels in soils (400 mg/kg) (Rodriguez and Fraga, 1999). Cao et al. (2003) reported that phosphate fertilizer increased soil arsenic availability to *P. vittata* grown in arsenic contaminated soils. These results show both arsenic and phosphorus are key factors impacting plant biomass and metal concentration in phytoremediation.

It is well known that a considerable number of bacterial species, mostly those associated with the rhizosphere, are able to exert a beneficial effect upon plant growth, by either providing plant nutrients or growth promoters. They are called 'plant growth promoting rhizobacteria (PGPR), however, usually their numbers are not high enough to compete with other bacteria commonly established in the rhizosphere (Rodriguez and Fraga, 1999). Therefore, their impact on plant growth is limited. Inoculation of plants by a target microorganism at a much higher concentration than that normally found in soil to outcompete with other non-PGPR is necessary to take advantage of the property of plant growth enhancement. By inoculating the fern by target

phosphorus and arsenic solubilizing microorganisms to outcompete with non-contributing communities, the efficiency of phytoremediation by *P. vittata* could be enhanced.

Several studies have been done to analyze the potential of microorganisms on arsenic accumulation by *P. vittata*; however, so far the efforts are mostly on abruscular mycorrhizal (AM) fungi. For example, AM fungi increase arsenic uptake by *P. vittata* when grown on arsenic-contaminated soils. This is attributed to enhanced plant P uptake and better plant growth. Both AM fungi isolated from arsenic-contaminated soils and those commonly found in soils such as *Glomus mosseae* and *Gigaspora margarita* functioned similarly. In addition, Liu et al.(2005) reported reduced plant arsenic uptake by *P. vittata* whereas others showed that AM fungi reduce *P. vittata* growth (Leung et al., 2006; Trotta et al., 2006). However, among the references cited here, there is no study focuses on the plant growth promoting bacteria.

1.5 Objectives

The overall hypothesis for this study is that bacteria in arsenic contaminated sites have evolved strong arsenic detoxification abilities, including those are beneficial for the arsenic hyperaccumulator. The objectives are therefore to isolate phosphorus solubilizing and arsenic-resistant bacteria from arsenic-contaminated soils and understand the mechanisms of their arsenic resistance.

Efforts were made to isolate PGPR associated with *P. vittata*, specifically, phosphorus and arsenic solubilizing bacteria, which can potentially enhance phytoremediation through improving metal removal rate and increasing harvested biomass. We identified and purified a group of the most As-resistant bacteria, which tolerated up to 30,000mg/kg of arsenic in liquid culture, the higher level reported to date. Experiment results suggested that arsenic resistance in those bacteria resulted from their efficiency in arsenic transformation and sequestration and their ability in scavenging oxidative burst and counteracting with different osmotic stresses.

CHAPTER 2
MATERIALS AND METHODS

2.1 Soil Sampling and Arsenic Concentration Analysis

A total of 12 soil samples (bulk and rhizosphere) and 3 plants were collected from two arsenic contaminated sites where *P. vittata* grew naturally in south Florida in April of 2007. The first site (CCA site) was contaminated from chromated copper arsenate, which was previously used for pressure treat lumber from 1951 to 1962 (Komar et al., 1998). The second site (RES site) was a residential site in central Florida, where CCA treated woods were used for stairs and decks. Rhizosphere soil was defined as the soil attached to the roots, which was removed from the roots by shaking gently. The bulk soil was collected from site without plant's influence. Soil samples were kept at 4ºC.

For arsenic concentration analysis, soil and plant samples were air dried (22ºC), mixed thoroughly and digested by nitric acid / hydrogen peroxide (USEPA Method 3051) in a heating block (Environmental Express, Ventura, CA). Arsenic concentrations in the solutions were analyzed by graphite furnace atomic absorption spectroscopy (GFAAS, Perkin Elmer SIMMA 6000, Perkin-Elmer Corp., Norwalk, CT). Analysis was carried out in triplicates.

2.2 Bacterial Isolation and Enumeration by Total Heterotrophic Counting

To select arsenic-resistant bacteria, three soil samples from CCA site and two soil samples from RES site, with different arsenic concentrations were used. The CCA soils included two bulk soils (92.8 and 167 mg/kg As) and one rhizosphere soil (80.0 mg/kg As) and the RES soils included one bulk soil (12.9 mg/kg As) and one rhizosphere soil (28.2 mg/kg As).

Soil samples (0.3 g) were suspended in 10 mL of sterilized water and vortexed rigorously. Soil suspensions (2, 20, and 200 µl) were plated onto two agarose media: modified TYEG (1/10 strength) or NBRIP agarose medium. The TYEG medium contained 1g/L tryptone, 0.3g/L yeast extract, 0.5/L glucose and 1% agarose. The NBRIP agarose

media contained 5g glucose, 5g $MgCl_2 \cdot 6H_2O$, 0.25g $MgSO_4 \cdot 7H_2O$, 0.2g KCl, 0.1g $(NH_4)_2SO_4$; 10mg $FeSO_4$, 14mg nitric acid, and 1% agarose, which was spiked with inorganic or organic phosphorus sources (5g of phosphate rock: $Ca_{10}(OH)_2(PO_4)_6(F, Cl)$; or sodium phytic acid: $Na_{12}C_6H_6O_{24}P_6$). After 2 days, bacteria produced most single colonies among 3 inoculum volumes on the media were inoculated to the respective media agarose plates containing different levels of arsenic (10, 50 100, 200, 300 and 400 mM arsenic as sodium arsenate). Number of survived bacterial colonies was counted after two days of growth.

Since arsenic availability in agar media (semi solid) may be limited, bacteria grew on 400 mM arsenic agar plate were inoculated into 400 mM arsenic TYEG liquid medium to test bacterial arsenic tolerance in liquid culture. All bacterial incubations were conducted at room temperature (22ºC).

2.3 Bacterial Identification

The twelve most arsenic resistant bacteria were identified by 16s rRNA gene sequencing method. Bacterium genomic DNA were extracted with phenol, then phenol/chloroform/isoamyl alcohol (pH 8.0 25:25:1), and chloroform/isoamyl (24:1). DNA was precipitated in 3M sodium acetate (pH 5.2) and ice cold ethanol, pelleted by centrifugation, ethanol washed 3 times, and then resuspended in water. 16s rRNA genes were PCR amplified by primers 8F 5'-AGA GTT TGA TCC TGG CTC AG-3' and 1489R 5'-TAC CTT GTT ACG ACT TCA-3', PCR condition included: initial denaturation for 7 min at 95 ºC, 30 cycles of 95 ºC for 1 min, 51ºC for 1min, 72ºC for 1min, and a final extension step at 72ºC for 10 min, with 0.2 μM final primer concentration (Bruneel et al., 2006). PCR products were cloned by TOPO® TA Cloning™ Kit (Invitrogen Inc.) and sequenced by ICBR Sequencing Lab at University of Florida. 16s rDNA phylogenetic tree was constructed by using the BLAST (Altschul et al., 1990), clustalX (Jeanmougin et al., 1998), and Treeview program (Page, 1996).

2.4 Bacterial Growth Characterization under Arsenic and Osmotic Stress

Bacterial isolates, which tolerated to 400mM sodium arsenate in liquid culture, belonged to 8 different genera according to 16s rRNA sequences (*Naxibacter sp.* AH4*, Mesorhizobium sp.* AH5*, Methylobacterium sp.* AH6*, Enterobacter sp.* AH10*, Pseudomonas sp.* AH21, *Bacillus sp.* AH22*, Acinetobacter sp.* AH23 and *Caryophanon sp.* AH28) were chosen for the growth characteristics study. Their arsenic resistance mechanisms were examined by measuring their growth characteristics under arsenic or osmotic stresses in two experiments. The first experiment compared bacterial growth characteristics under same ionic strength using sodium arsenate or sodium chloride. The second experiment was conducted under -1.5M Pa of osmotic stress using 3 different sources: sodium arsenate, sodium chloride and polyethylene glycol 6000 (PEG 6000). Both experiments were conducted with three replicates.

In the first experiment, 2.4 M of NaCl or 400 mM of sodium arsenate were used to compare bacterial growth (Naydenov et al., 2006), with control bacteria growing in TYEG medium with no osmotic stress. Bacteria were grown at room temperature (22ºC) with constant agitation (150 rpm). Bacterial growths were measured at 0, 6, 20 and 26 hours by a spectrometer (SHIMADZA BioSpec-Mini) at 600 nm (OD_{600}) (1 cm light-path length) after inoculation.

The second experiment was done similarly except using 176 mM of sodium arsenate (calculated by MINTEQA2, (Allison et al., 1999)), 400 mM of NaCl or 26% of PEG (Sosa et al., 2005), which produced -1.5M Pa of osmotic stress. Bacterial growths were monitored over 42 hours. The extra time in the second experiment allowed comparing stationary phase growth and Cl^- toxicity in the isolates.

2.5 Oxidative Stress Test

Preliminary experiment was done to identify *Salmonella typhimurium* mutants. Strains of *S. typhimurium* SF1005 (unmarked *rpoS* mutation), *S. typhimurium cc1000 ropS*::*tet* and *S.*

typhimurium GS014 *oxyR*::Tn10 were tested using hydrogen peroxide (Aaron Industries Inc., SC, USA) and N,N'-Dimethyl-4,4'-bipyridinium dichloride (paraquat) (Sigma) either by measuring inhibition area on Petri dishes, or by measuring bacterial growth in 96-well cell culture plates.

Measuring inhibition area on Petri dishes. Aliquots of 50 µl of overnight-grown bacterial culture were plated in TYEG agar media and bacteria were allowed to grow for 4 hours. A 6 mm sterile paper disc was placed on each plate, to which 25 µl of 3% hydrogen peroxide or 50 mmol/L of paraquat were applied. Plates were incubated at 22ºC, and the inhibition zone of bacterial growth was measured after 24 and 48 hours. The experiments were carried out in triplicates.

Measuring bacterial growth in 96-well cell culture plates. Bacterial isolates were inoculated to R2A medium (proteose peptone 0.5g/L, casamino acids 0.5 g/L, yeast extract 0.5 g/L, glucose 0.5 g/L, soluble starch 0.5 g/L, K_2HPO_4 0.3 g/L, $MgSO_4 \cdot 7H_2O$ 0.05 g/L, sodium pyruvate 0.3 g/L and 1.5% of agarose, final pH7.2 at 25 ºC) to starve the cells. Cells were scratched from the surface of the plate using cotton swab, then suspended in M9 minimal medium (12.8g $Na_2HPO_4 \cdot 7H_2O$, 3g KH_2PO_4, 0.5g NaCl, 1.0g NH_4Cl, 2mM $MgSO_4$, 100µM $CaCl_2$) with 0.02% of glucose as carbon source and 0.03% of tetrazolium violet (TV) (Sigma) as a bacterial respiratory indicator. TV was stocked in ethanol stock solution. 300µl of final volume with 50mM of paraquat was used to test in 96 well plates. Initial cell concentration was OD_{590}=0.02. Bacterial growth was determined by measuring absorption of suspension at 590 nm after 24 hours (Tracy et al., 2002). *S. typhimurium* LT2 oxyR::Tn10 and 14028 wild type were used as controls after checking the growth of all the mutations.

2.6 Arsenic Transformation by Arsenic Resistant Bacteria

Arsenic transformation by three arsenic-resistant bacterial isolates (*Naxibacter sp.* AH4, *Mesorhizobium sp.* AH5, *Pseudomonas sp.* AH21) and a non-arsenic resistant bacterium *Sinorhizobium meliloti* MG32 were analyzed. The experiment included a control without

bacterium, and 3 replicates for each treatment. Bacterial medium contained either 1mM arsenite or arsenate and started at same cell density (OD_{600} = 0.1). Cell cultures were grown at room temperature with 150 rpm constant shaking. Shake cultures were sampled after 4, 8, 16 and 32 hours. Total arsenic was analyzed by a graphite furnace atomic absorption spectrophotometer (240Z, Varian, Walnut Creek, CA). As speciation was done by arsenic speciation cartridge (Metal Soft Center, Highland Park, NJ) (Meng et al., 2001).

2.7 Arsenate Reductase Assay

Cell culture of three arsenic-resistant bacteria *Naxibacter sp.* AH4, *Mesorhizobium sp.* AH5, *Pseudomonas sp.* AH21and control bacteria *Escherichia coli* DH5α were grown in TYEG and LB media overnight, respectively, before inoculated into 50 mL of TYEG or LB media. Protein expression was induced by 0.1 mM sodium arsenate after the lag phase (about 3 hours), then shaken for another 4 hours before harvesting the cells by centrifugation. Cell pellets were resuspended in reaction buffer (10 mM Tris, pH 7.5, 1 mM Na_2EDTA, 1 mM $MgCl_2$, 1 mM DTT) (Anderson and Cook, 2004) with 0.1 mM of proteinase inhibitor phenylmethylsulfonyl fluoride (PMSF, SIGMA); Total proteins were extracted by glass beads and centrifugation in 4ºC, and the concentration was determined by Bio-Rad Protein Assay (Bio-Rad Laboratories, USA). Arsenate reductase assay solution contained 150 µl protein extracts, 1 ml reaction buffer, 10 µM sodium arsenate, 0.5 mM NADPH, 1 mM GSH, 2U yeast glutathione reductase and 0.02µM *E. coli* Glutaredoxin 2. Enzyme activity was determined by measuring the absorption at 340 nm (Gladysheva et al., 1994).

2. 8 Arsenic Detoxifying Gene Determination

The details for degenerated or regular primers for *arsR*, *arsB* and *arsC* gene amplifications were described below: *arsR* forward: 5'-ATGMTYAMCCCTCCCCARGTCTTYAAAT-3' and *arsR* reverse: 5'-TYAACAACAASCGGCKGCGCGCW-3'. PCR condition included: initial denaturation for 7 min, 40 cycles of 94ºC for 45s, 58ºC for 30s, 72ºC for 1min, and a final extension step at

72ºC for 10 min, with 0.2 µM final primer concentration (Mandal et al., 2008); *arsB* forward: 5'- ATGGCAACCGAAAGGTTTAG-3', *arsB reverse:* 5'-GTTGGCATGTTGTTCATAAT- 3'. PCR condition was: 94°C for 7 min and 29 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 10 min (Anderson and Cook, 2004), with 0.2µM of primers; *arsC* forward: 5'-GCATTCTTTCCGAAGCCATGTTCAA- 3', *arsC* reverse: 5'-AGCTCACGCTTGAGCTGGTCGCGAT- 3', which were designed based on *Pseudomonas aeruginosae* PAO1 *arsC* gene. A *Pseudomonas aeruginosae* PAO1 strain was used as a positive control for both *arsB* and *arsC,* and the PCR products from this organism were confirmed by sequencing and then used as probes for Southern hybridization. PCR condition was: 94°C for 7 min and 29 cycles at 94°C for 1min, 56.8°C for 1mins, 72°C for 1 min, and a final extension at 72°C for 10 min, with 0.2µM of primers. Southern Blot followed the method of Russell (2001) and user manual of DIG DNA labeling & Detecting Kit (Roche Diagnostics, USA. REF: 11093657910 Cat: 1093657). Primers for *arrA* gene cloning were *ArrA* forward: 5'- AAGGTGTATGGAATAAAGCGTTT gtbgghgaytt- 3' and *ArrA* reverse: 5'- CCTGTGATTTCAGGTGCCcaytyvggngt-3') (lower case indicated the degenerated parts). *Shewanella sp.* ANA3 was used as *arrA* positive control. PCR reactions included incubation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 50°C for 40 seconds, and 72°C for one minute. The final primer concentration was 0.5 µM (Malasarn et al., 2004)**.**

2. 9 Arsenic Detoxifying Plasmid Determination

Arsenic resistant isolates were divided into 10 groups and each group containing 5 mL of mixed cell culture in TYEG medium was used for plasmid extraction. Plasmid extraction performed by the guideline of QIAprep Spin Miniprep Kit (QIAGEN Science, Maryland, USA). Plasmids were transformed into chemical competent *E. coli* DH5α and plated on LB media containing 50 mM sodium arsenate to test their arsenic tolerance. Plasmid in As^{-r} *E.*

coli DH5α was further isolated and transformed again into *E. coli* DH5α to confirm plasmid encoded arsenic resistant functional genes. Isolates in groups with As^{-r} plasmid were further tested one by one to identify where the plasmid was from.

2.10 Antibiotics Test

All identified isolates were tested with their kanamycin resistance at 50μg/ml in TYEG with 1% of agarose plates. Negatives results further tested their resistance with carbenicillin, nalidix acid, ampicillin and neomycin at concentration of 50 μg/ml.

2.11 *Mesorhizobium sp.* AH5 Genomic DNA Library Construction

Bacterial isolate *Mesorhizobium sp.* AH5 was chosen to construct a DNA library to search for functional genes contributing to arsenic resistance. An amount of 1.5μg bacterial DNA was extracted and partial digested with EcoRI or BamHI at 37°C for one hour. Digestion was stopped by loading on ice for 5 minutes. DNA fragments at about 10 thousand base pairs were purified by Gel Band Purification Kit (GE Healthcare, USA) and ligated with CopyControl Vector (CopyControl™ BAC Cloning Kit, EPICENTR, Wisconsin). The vector was then transformed into competent *E. coli* DH5α and selected with LB plates added with 40mg/ml of x-gal, 0.4mmol/L of Isopropyl β-D-1-thiogalactopyranoside (IPTG) and 12.5μg/mlof chloramphenicol. Positive colonies were inoculated into plates containing 50mM of sodium arsenate.

GenBank Accession Numbers of the 16S *rRNA* gene sequences of the isolates: AH4: FJ621305; AH5: FJ621306; AH6: FJ621307; AH10: FJ621308; AH21: FJ621309; AH22: FJ621310; AH23: FJ621311; AH25: FJ621311; AH28: FJ621313; AH34: FJ621314; AH43: FJ621315; AH45: FJ621316.

CHAPTER 3
RESULTS AND DISCUSSIONS

3.1 Arsenic Concentrations in Soil and Plant Samples

Total arsenic concentrations in soil and *Pteris vittata* samples from two contaminated sites (CCA and RES) are showed in Figure 3-1. The data show that soils were heterogeneous with varying arsenic concentrations in the contaminated sites, with rhizosphere soils generally containing higher arsenic concentration than those in bulk soils.

Arsenic concentrations in the fronds of *P. vittata* were substantially higher than those in the roots. Translocation factor (TF), which is defined as the ratio of metal concentrations in the fronds to those in the roots, was 31.7 for plants growing in CCA soil, and 14.7-21.9 for plants growing in RES soil. Bioaccumulation factor (BF), which is defined as the ratio of metal concentration in plant biomass to that in the soil, ranged from 21.5 of the CCA plant, to 171, 110 of the two RES plants. While TF has been used to determine the effectiveness of plants in translocating metals from the roots to the shoots, BF has been used to determine the effectiveness of plants in removing metals from soils.

3.2 Arsenic Resistant Bacteria from Contaminated Soils

Culturable bacteria account for only less than 1% of natural bacterial communities (Amann et al., 1995). Techniques exploring phospholipid fatty acid (PLFA) or nucleic acids such in terminal restriction fragment length polymorphism (TFLP), denaturing gradient gel electrophoresis (DGGE) are more robust in unveiling microbial community structures. However, arsenic resistance bacteria are special case.

It is known that the formation of microbial biofilm protects bacterial community from toxic agents. Although there are no direct data showing formation of biofilm help bacteria resist higher arsenic concentrations, it has been showed that antimicrobial agents such as chlorine and many antibiotics failed to penetrate the biofilm (Mah and O'Toole, 2001). Therefore, it is reasonable to assume that formation of microbial biofilm may also protect

bacterial community from arsenic toxicity. Due to the heterogeneous property of soil, microbial communities in contaminated sites are not necessarily having higher ability in arsenic detoxification. Therefore, obtaining pure bacterial culture is still the first choice in selecting environmental bacteria possessing the ability in arsenic detoxification.

In this experiment, arsenic-resistant bacteria were isolated from two arsenic-contaminated soils (CCA and RES), which were plated on two media TYEG and NBRIP containing up to 400 mM arsenate. In both sites, *P. vittata* rhizosphere soils sustained substantially higher number of arsenic resistant bacteria, compared to bulk soils with similar arsenic concentrations (Table 3-1, 3-2). For example, in CCA site, 42.5% of the tested colonies were able to grow in TYEG containing 400mM of arsenate, while the numbers in the two bulk soils from the same site were 16.9% and 3.4%, respectively, all starting with about 80 single colonies (Table 3-1). In RES site, the percentages of survivals in 400 mM TYEG plates were 13.3% versus 3.1% for rhizosphere soil and bulk soil respectively. Totally fifty-three isolates were obtained from plates containing 400mM of arsenate, and designated as AH1 to AH53. Among them, AH1 to AH4 were isolated by NBRIP and the rest were by TYEG medium.

Previous study revealed that majority of arsenic that *P. vittata* took up were solubilized from recalcitrant fractions in soil (51-71% were from Ca-bound arsenic) (Fayiga et al., 2007). Therefore, rhizosphere bacteria may be exposed to higher bioavailable arsenic concentration than those from bulk soils. Under this selective pressure for decades, microbial community developed sophisticate arsenic detoxification mechanisms to overcome the growth restrictions. In Table 3-1 and 3-2, arsenic resistant bacteria presented as percentage of the bacterial colony number tested, which minimizes the effects of nutrition and soil heterogeneity. The fact that higher percentage of arsenic resistant bacteria were from rhizosphere soils indicated that, during plant arsenic accumulation, higher bioavailable

arsenic mobilized from soil induced higher ability of arsenic toleration in the associated microbial community.

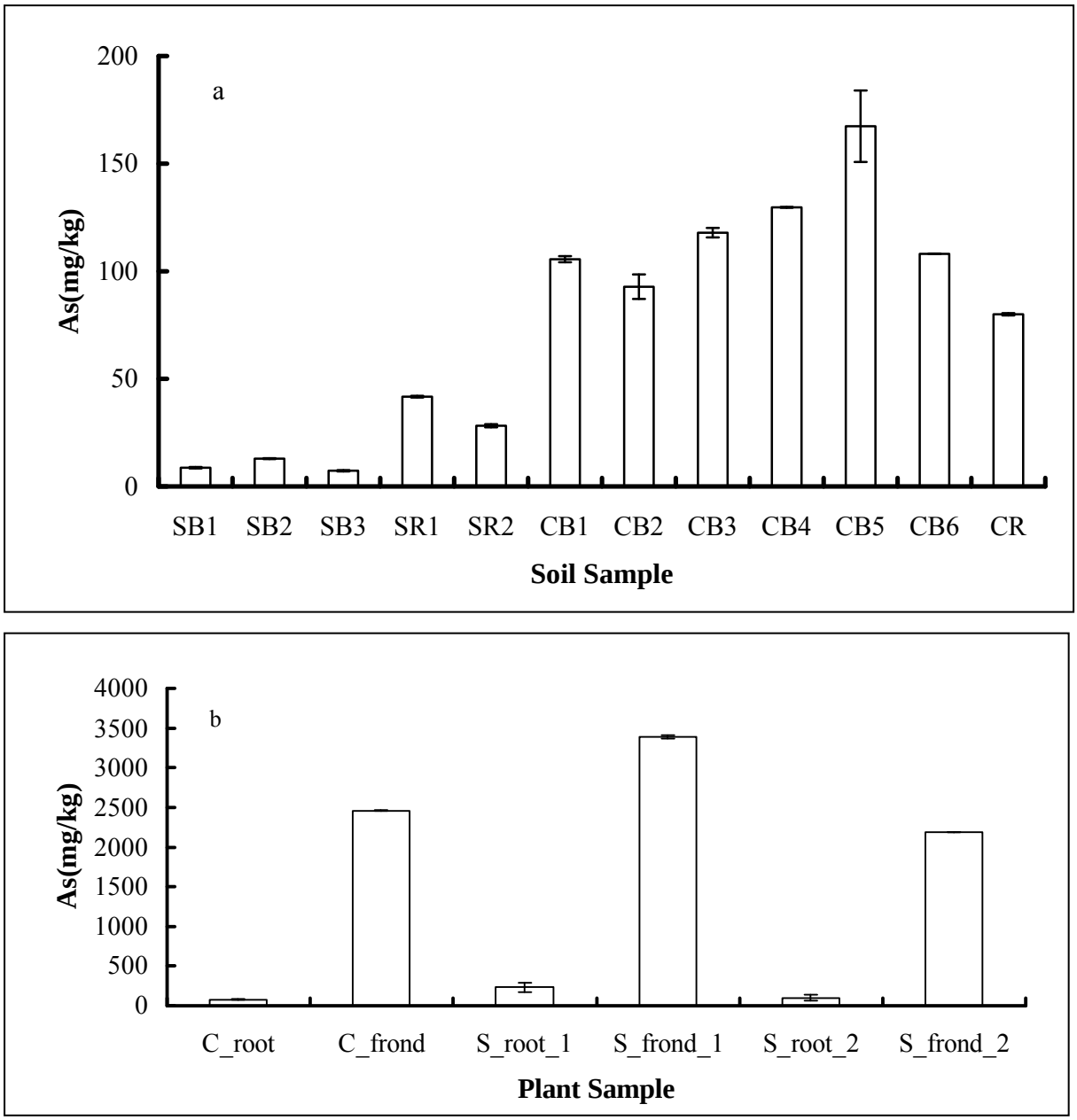


Figure 3-1. Arsenic concentrations in soil (a) and *Pteris vittata* (b) samples from two arsenic contaminated sites. S: RES site; C: CCA site; B: bulk soil; R: rhizosphere soil. Two plants from RES site and one plant from CCA site were analyzed. Soil_53 is standard soil sample with 151.0 mg/kg of arsenic. Points are means and standard errors of 3 replicates.

3.3 Phosphorus Solubilizing Bacteria from Arsenic Contaminated Soils

A group of phosphorus solubilizing bacteria was isolated. Those bacterial isolates were capable of mobilizing phosphorus from either organic or inorganic sources. They were also

arsenic tolerant (Table 3-2), though not as tolerant as those cultured in TYEG media. Those bacteria can used to increase P availability in agricultural soils as well as arsenic contaminated soils to improve plant's P nutrition.

Phytic acid has been identified as one of the major compounds of *P. vittata* root exudates (Tu et al., 2004). In addition, phosphorus has been identified to play a major role in arsenic detoxification by *P. vittata*. Only several bacterial colonies, which were identified as *Naxibacter sp.* AH1 and AH4, were able to grow on NBRIP with phytic acid. They were isolated from CCA 3R and RES 5R, showing the existence of organic phosphorus solubilizing bacterial communities in rhizosphere soil (Figure 3-2). Two of them with unique yellow and red pigments were identified as *Naxibacter* species by 16s rDNA sequence method (Sequences are available in Appendix A).

Figure 3-2 shows the two bacteria on NBRIP plates, with their 16s rRNA gene sequences sharing 98% of similarity. Bacterium AH1 produced red pigment (A) and was more sensitive to arsenic, which can't survive in 400 mM of sodium arsenate (data not shown). *Naxibacter* is a recently described bacterial genus by Xu et al.(2005) and at present the genus comprises only one species, *Naxibacter alkalitolerans* (Kampfer et al., 2008). However, 16s rRNA gene of bacteria AH1 and AH4 shared <97% of similarity with the published *Naxibacter* species, which is a border line to define a species by 16s rRNA. It is possible that AH1 and AH4 are new bacteria that have not been fully described.

However, no bacteria were recovered on NBRIP media without additional glucose as C source, showing that no chemoheterotrophic bacterium utilizing phytic acid as carbon source was isolated from the soils, although bacteria were able to use phytic acid as P source.

Compared to the large number of arsenic-resistant bacteria recovered by TYEG medium with arsenic concentrations up to 400 mM in agar plates, those bacteria able to use organic or inorganic phosphorus from the immobilized sources (phytic acid or rock phosphate)

in NBRIP medium were much less in quantity and much more sensitive to arsenic. Table 3-2 shows percentage of arsenic resistant bacteria among those isolates that were able to use rock phosphate as phosphorus source. This may imply that bacterial ability to solubilize P was not related to its ability to tolerate arsenic or the bacteria couldn't obtain sufficient amount of P to help detoxify arsenic since P availability in phosphate rock was much lower than that provided in TYEG growth media. However, one of phosphorus solubilizing bacteria AH4 was identified as one of the 12 most arsenic resistant bacteria (Table 3-3).

Table 3-1. Percentage of arsenate resistant colonies recovered in TYEG medium isolated from different soils and under different arsenic concentrations*.

Soil used	Soil As mg/kg	Arsenic concentrations in TYEG medium (mM)					
		10	50	100	200	300	400
CCA 1B*	92.5	62.3	46.8	42.9	40.3	36.4	16.9
CCA 2B	167	40.9	37.5	36.4	21.6	15.9	3.40
CCA 3R	80.0	68.1	66.0	57.4	46.8	42.5	42.5
RES 4B	12.9	15.9	9.50	4.80	3.10	3.10	3.10
RES 5R	28.0	78.7	76.0	74.7	72.0	69.3	13.3

Table 3-2. Percentage of bacteria survived in NBRIP + phosphate rock plates under different arsenic concentrations

Soil used	Soil As mg kg^{-1}	Arsenic concentrations in NBRIP medium (mM)					
		10	50	100	200	300	400
CCA 1B*	92.5	87.2	38.5	25.6	15.4	5.1	0
CCA 2B	167	98.9	73.3	14.4	4.40	0	0
CCA 3R	80.0	54.3	F*	20.0	20.0	0	0
RES 4B	12.9	0	F	0	0	0	0
RES 5R	28.0	53.7	F	24.1	0	0	0

* B= bulk soil; R= rhizosphere soil; CCA site = chromated copper arsenate contaminated site, and RES = residential contaminated site;
*F: No data, media precipitation.

Figure 3-2. *Naxibacter sp.* AH1 (A) and *Naxibacter sp.* AH4 (B) on agarose plates.

3.4 Characterization and Identification of Arsenic Resistant Bacteria

Rhizosphere soil is typically aerated and nutrient rich. This experiment attempted to isolate chemoheterotrophic bacteria from rhizosphere, which were able to tolerate high concentration of arsenic. However, the method didn't preclude that the isolates would also be facultative anaerobics. Totally, 53 isolates that can grow on plates containing 400mM arsenate were obtained from two CCA contaminated sites. These strains were designated as AH1 to AH53. Because TYEG is nutrient-rich medium and NBRIP is defined medium, bacteria recovered by NBRIP were all able to grow in TYEG. Therefore, for arsenic resistant mechanism study, TYEG was used for all experiments.

Considering that arsenic availability in agarose plates is lower than that in liquid culture, 53 isolates were then screened in TYEG liquid culture containing 400 mM sodium arsenate. Those were able to grow were further separated and identified based on 16s rRNA analyses (Table 3-3). Based on the BLAST results of the 16s rRNA gene, the 12 most arsenic resistant soil isolates were identified as *Naxibacter sp.* AH4, *Mesorhizobium sp.* AH5, *Methylobacterium sp.* AH6, *Enterobacter sp.* AH10, *Pseudomonas sp.* AH21, *Bacillus sp.* AH22, *Acinetobacter sp.* AH23, *Pseudomonas sp.* AH25, *Bacillus sp.* AH28, *Pseudomonas sp.* AH34, *Pseudomonas sp*. AH43, and *Pseudomonas sp.* AH45.

A phylogenetic tree of the isolates and references from GenBank were shown in Figure 3-3. The references in Figure 3-3 were all from culturable isolates and well identified. GenBank access numbers and DNA sequences are available in Appendix B.

In this study, total heterotrophic arsenic resistant bacterial counts from arsenic contaminated sites showed a higher quantity and quality of arsenic toleration in rhizosphere soil compared to bulk soils (Table 3-1, 3-2). The 8 genera of the 12 most arsenic resistant bacteria belong to the two phyla: Proteobacteria and Firmicutes (Figure 3-3). Consistent results are found in literature, which show that majority of the 16S rRNA gene sequences of cultured prokaryotes are from Proteobacteria (ca. 50%), Actinobacteria, Firmicutes (ca. 15%), and Bacteroidetes within GenBank (Riesenfeld et al., 2004). Therefore, the isolates in this study represent the cultivable aerobic heterotrophic, which are arsenic resistant.

Table 3-3. The 12 most arsenic-resistant bacteria identified from two arsenic contaminated soils

ID	CCA/RES* site	R/B*
Naxibacter sp. AH4	RES	R
Mesorhizobium sp. AH5	CCA	B
Methylobacterium sp. AH6	CCA	B
Enterobacter sp. AH10	CCA	B
Pseudomonas sp. AH21	CCA	R
Bacillus sp. AH22	CCA	R
Acinetobacter sp. AH23	CCA	R
Pseudomonas sp. AH25	CCA	R
Bacillus sp. AH28	CCA	R
Pseudomonas sp. AH34	CCA	R
Pseudomonas sp. AH43	RES	R
Pseudomonas sp. AH45	RES	R

*CCA: chromated copper arsenate contaminated site; RES: residential contaminated site; R: rhizosphere soil; B: bulk soil.

Up to date, *Corynebacterium glutamicum* was the only known microorganism, which tolerated up to 400 mM sodium arsenate (Mateos et al., 2006). *C. glutamicum* is in the phylum of Actinobacteria, G^+, aerobic or facultatively anaerobic. In fact *C. glutamicum* is one of the biotechnologically most important bacterial species in use today. The group of arsenic resistant bacteria isolated in this study are either able to solubilize phosphorus or arsenic, or are potentially beneficial bacteria for arsenic phytoremediation by *P. vittata*. In addition, they also had the potentials for bioleaching to remove arsenic or phosphorus from soils.

Tn5 transposon mutation library has been used to search for bacteria functional genes. However, in using Tn5 transposon, bacteria need to be sensitive to kanamycin. Most arsenic resistant bacteria should have antibiotic resistance, so their antibiotics resistant profiles were examined. The results in Table 3-4 showed that, except for *Mesorhizobium sp.* AH5, *Pseudomonas sp.* AH36, and *Pseudomonas sp.* AH47 (identified by 16s rRNA gene sequences, sequence are listed in Appendix A), all arsenic resistant bacteria identified in this study carried kanamycin resistant genes. The resistant profiles of the 3 bacteria were shown in Table 3-4. *E. coli* DH5α is an experimental control. The results showed broad antibiotics resistance in arsenic resistant bacteria.

Tn5 transposon mutation library should be carried out in future study based on the results on bacterial genomic DNA library in *E. coli* DH5α. Indeed, the two methods represent the concepts of forward genetics and reverse genetics. Because different arsenate reductase genes require different cofactors or other host differences, there is no guarantee that functional genes can be found by forward genetics of genomic DNA library in *E. coli* DH5α. Therefore, the reverse genetics of a Tn5 transposon mutation library may be another approach.

Table 3-4. Antibiotic resistance test on the arsenic resistant bacteria isolated from arsenic contaminated soils

Antibiotic	*Mesorhizobium sp.* AH5*	*Pseudomonas sp.* AH36	*Pseudomonas sp.* AH47	*E. coli* DH5α
Carbenicillin	+	+	+	-
Nalidix acid	+	+	+	-
Ampicillin	+	+	+	-
Neomycin	+	+	+	-
Kanamycin	-	-	-	-

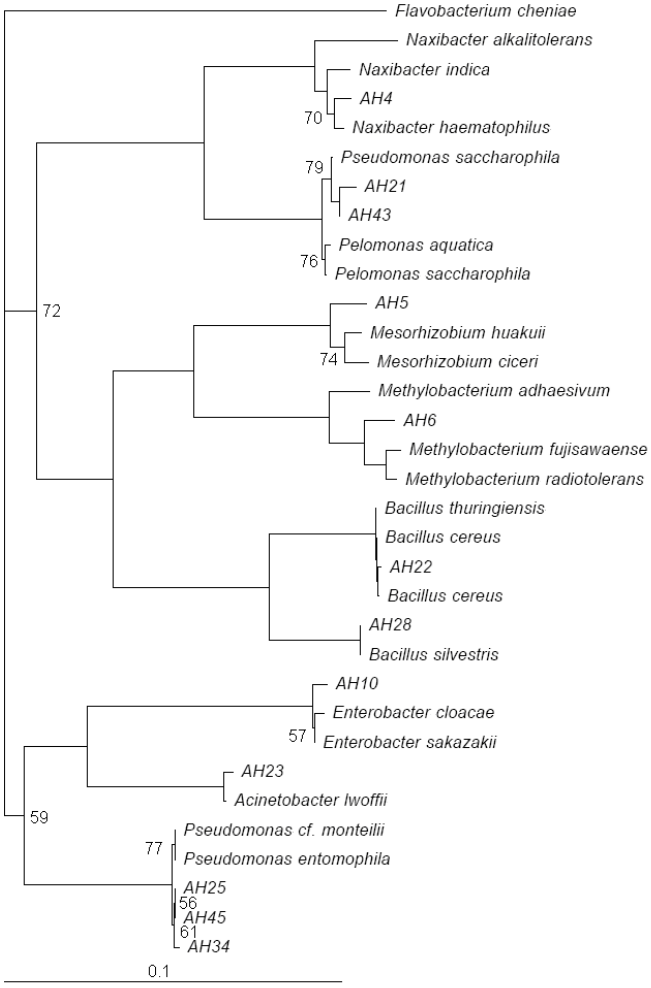


Figure 3-3. Phylogenetic tree of the 12 most arsenic resistant bacteria based on 16s rRNA sequences.

F. cheniae 16s rRNA gene obtained from GenBank was used as an outgroup (Accession no. EF407880). Numbers represent percentages of 1000 bootstraps and are shown only for bootstrap value<80%. The scale bar represents 1 nucleotide substitution per 100 nucleotides of 16s rRNA sequence.

3.5 General Stress Tolerance Characteristics of Arsenic Resistant Bacteria

3.5.1 Growth Characteristics under Arsenic Stress

The growth characteristics of eight most arsenic-resistant bacteria were compared in the presence of 0.4 M sodium arsenate or 2.4 M NaCl (with similar ionic strengths) to determine if bacteria's arsenic resistance was related to salt tolerance. All eight strains had a longer lag phase in 0.4 M sodium arsenate comparing to the growth curve in TYEG medium, and the exponential growth started after 6 hours (Figure 3-4). Except for *Naxibacter sp.* AH4, which had a higher OD_{600} in TYEG medium with 2.4 M NaCl than 0.4 M sodium arsenate, all the other strains were not able to grow at such high NaCl concentration. This result indicted that they were not tolerant to such high salt concentration. In comparison, after 25 hours, there was little difference in growth between arsenic and TYEG treatment.

The fact that the growth curve as measured by OD_{600} after 26 hours under arsenate stress in strain AH5, AH10 and AH28 were almost the same as the TYEG control indicated limited toxicity effect of arsenate on the isolates. This was confirmed in the follow up experiment, where bacteria were grown in -1.5MPa osmotic stress. After 25 hours, there was little difference in growth between As and TYEG treatment (Fig. 3-6). This result also showed a higher OD_{600} in sodium arsenate than NaCl or PEG (Figure 3-6). Bacteria also grew better in NaCl than under same osmotic stress of PEG.

Arsenic resistant index (ARI), which was defined as the ratio of the growth rate in medium with 400 mM arsenic to that in the control medium, was developed to normalize bacterial arsenic detoxification (Figure 3-5). The closer ARI is to 1, the smaller toxicity of arsenic is to the soil bacteria. The ARI index showed that, *Mesorhizobium sp.* AH5, *Enterobacter sp.* AH10 and *Bacillus sp.* AH28 were the most efficient in arsenic detoxification (α= 0.01) and their growths were only less that 30% slower in 400 mM of sodium arsenate than TYEG without arsenic. The ability of the soil isolates in arsenic detoxification was also shown in the osmotic stress test. Though the control strain *P.*

fluorescens CHAO was able to grow at -1.5M Pa of osmotic stress, it didn't grow under arsenic stress (Figure 3-6).

Those bacteria were shown to be efficient in arsenate reduction (Fig. 3-10). Under the model of arsenate reduction detoxification under aerobic condition, bacteria take up arsenate through phosphate transporter, and reduce it to arsenite by specific enzyme with the help of glutaredoxin, thioredoxin or ferredoxin, and arsenite is then extruded out by membrane pumper (Rosen, 2002). The efficiency of phosphate transporter is positively correlated with cellular growth; after normalized with cellular growth, ARI indicates the comprehensive effects of bacteria's ability in reducing and extruding arsenic in this model. The fact that bacterial isolates *Mesorhizobium sp.* AH5, *Enterobacter sp.* AH10 and *Bacillus sp.* AH28 had the highest score of ARI indicates their highest ability in arsenic detoxification.

In this study, we proposed, for the first time, the model of multiple levels of arsenic tolerance. In other words, while specific functional enzymes take up, reduce and extrude arsenic, global gene regulations are turned on to counteract arsenic-induced growth stresses, such as oxidative burst or osmotic stress. From the angle of overall cellular metabolisms, ARI also indicates bacteria's ability in overcoming cellular toxicity. The closer ARI is to 1, the higher its ability in scavenging oxidative burst and osmotic stress that the arsenic resistant bacteria have. For example, the ARI of *Methylobacterium sp.* AH6 is 0.29, which means overall cellular metabolism was reduced by 71% from arsenic toxicity. The ARI of *Bacillus sp.* AH28 was 0.75, suggesting that 25% of cellular metabolism was spent on arsenic detoxification.

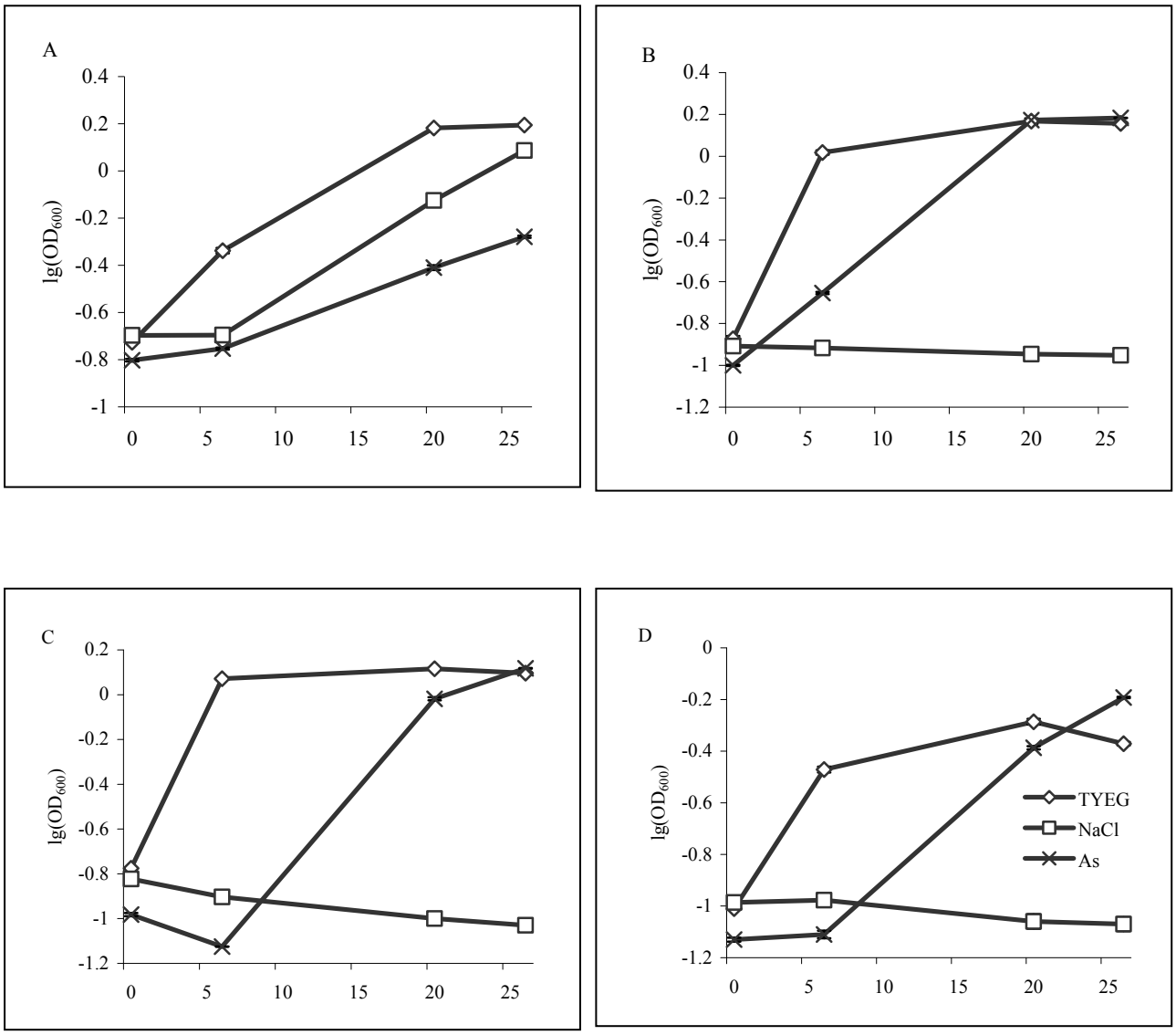


Figure 3-4. Growth characteristics of eight arsenic-resistant bacteria in TYEG medium containing 400 mM sodium arsenate or 2.4 M sodium chloride. The legend diamond denotes TYEG medium without additional salts, rectangle denotes TYEG with 2.4 M of NaCl, and cross denotes TYEG medium with 400 mM of sodium arsenate. A: *Naxibacter sp*. AH4; B: *Mesorhizobium sp.* AH5; C: *Methylobacterium sp*. AH6; D: *Enterobacter sp*. AH10; E: *Pseudomonas sp.* AH21; F: *Bacillus sp.* AH22; G: *Acinetobacter sp.* AH23; H: *Caryophanon sp.* AH28. Points are means and standard errors of 3 replicates.

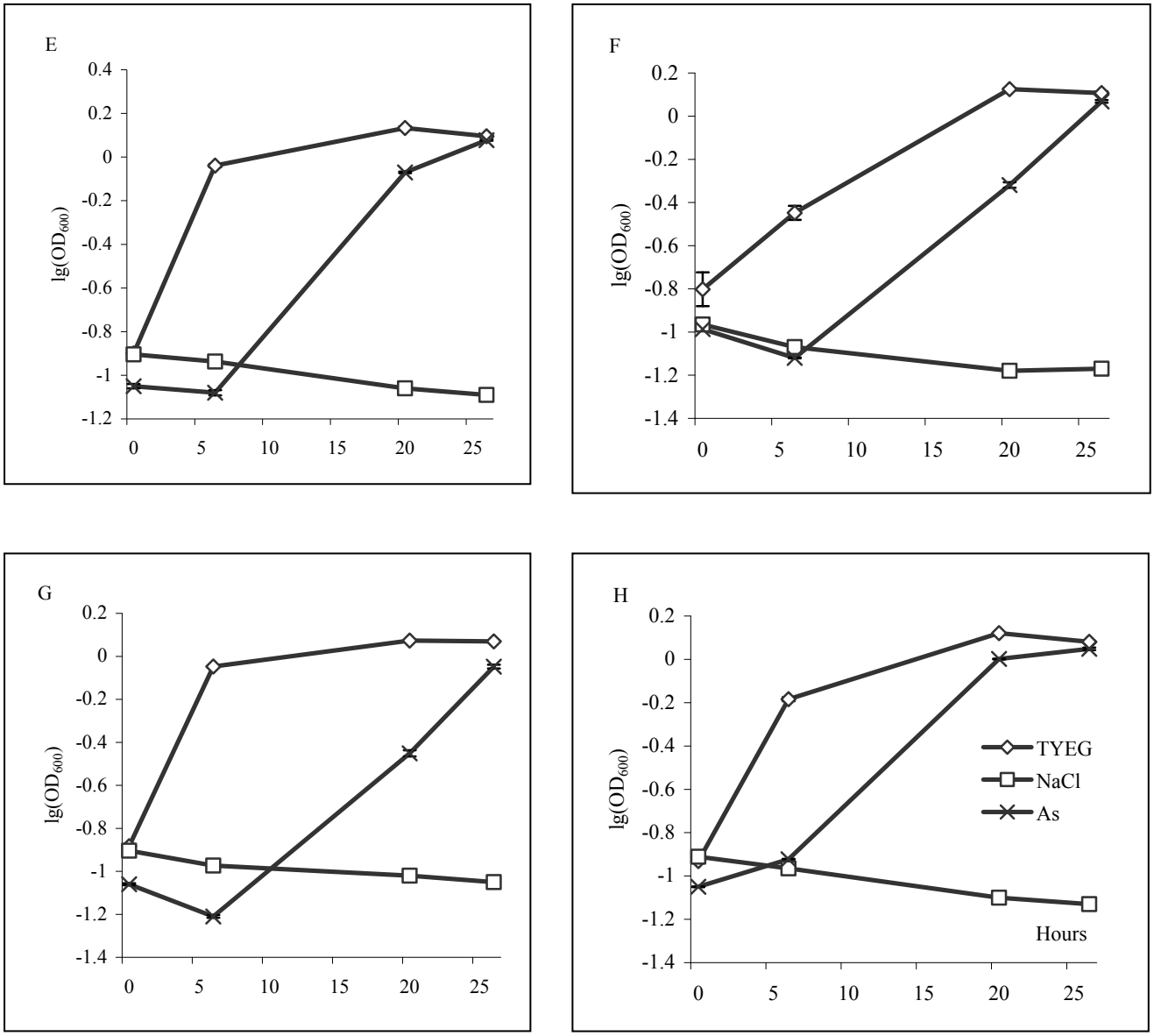


Figure 3-4. Continued.

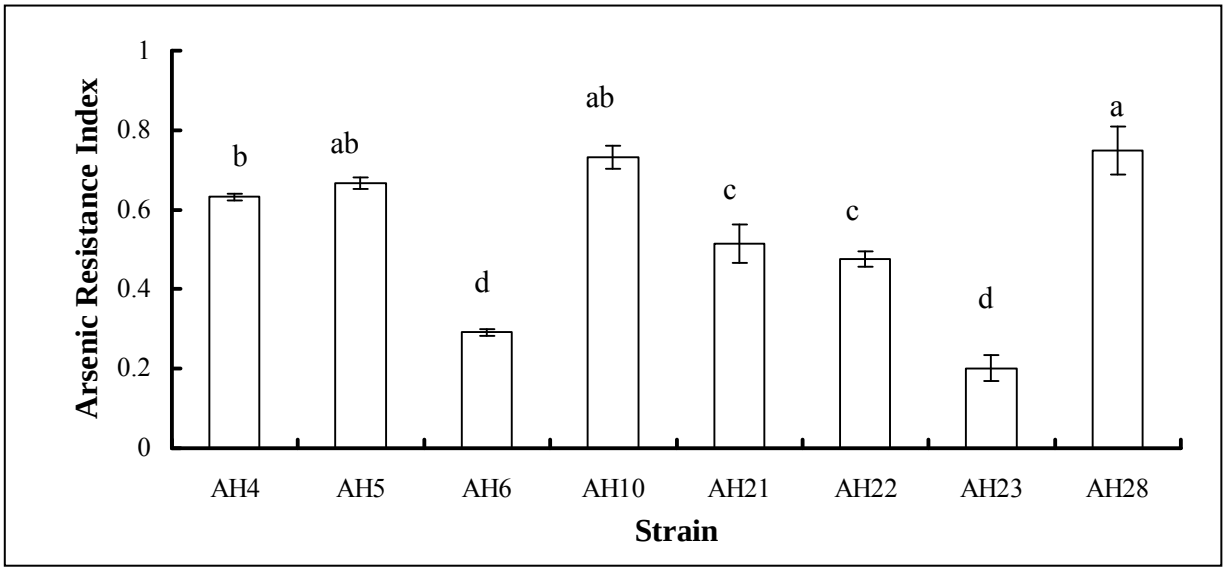


Figure 3-5. Arsenate Resistant Index (ARI) based on bacterial growth at 400 mM sodium arsenate (α= 0.01). ARI is defined as the ratio of the growth rate in medium with 400 mM arsenate to that in the control medium. Columns are means and standard errors of 3 replicates.

3.5.2 Arsenic Resistant Bacterial Growth under Osmotic Stress

None of the isolates were able to grow in NaCl under similar ionic strength of 400 mM of sodium arsenate except AH4 (Figure 3-4). Instead of using lethal dose of NaCl, the second growth experiment used -1.5M Pa of osmotic stress that the eight arsenic-resistant bacteria can tolerate to, which was equal to 400 mM of NaCl, 26% of polyethylene glycol 6000 (PEG6000) and 176 mM of sodium arsenate (Allison et al., 1999; Sosa et al., 2005). Growth results showed that the isolates grew better in NaCl than PEG at the same osmotic stress level (Figure 3-6).

All bacteria except *P. fluorescens* CHAO grew in the presence of sodium arsenate, exhibiting their tolerance to high arsenic concentration (Figure 3-6). All the strains except *P. fluorescens* CHAO were tolerant to -1.5MPa or higher osmotic stress, which was proved by growth in the presence of NaCl and PEG. With the same amount of inoculation, the initial absorption in medium with PEG was smaller than TYEG, NaCl or arsenate in all strains. This suggested that the impermeable solute reduced cell absorption or led to cell break down.

However, bacteria AH10 and AH22 grew better in PEG than NaCl at the end of the experiment.

Compared to TYEG, NaCl and PEG, there was a lack phase for arsenic resistant bacteria to grow in the presence of arsenic, indicated that some of the detoxification related genes were not housekeeping genes. Exponential growth started 6 hours after inoculation except for bacteria AH22 and AH23, which was because that, while others were inoculated from fresh cell culture, the two strains were inoculated from cultures that had been kept in 4 ^{o} C. After 25 hours, there was little difference in growth between NaCl, PEG and TYEG treatment. After 35 hours, bacteria in the medium with arsenic all reached to stationary phase. After 45 hours, all bacteria grew better with arsenic than with NaCl or PEG except AH23.

NaCl and PEG represent two type of osmotic stress: salt and water stresses. Both water and salt stress inhibited cellular growth to some extent and they have a common effect, lowering the osmotic potential of the cellular environment. While water stress is caused by using impermeable solutes (such as PEG 6000), salt stress is caused by various ions, mainly Na^{+} and Cl^{-}, which can be transported into and out of cells. Thus, salt stress has additional specific effect of ions present in the environment. The fact that arsenic resistant bacteria grow better in NaCl indicates the existence of cross protection between arsenic and other ion generated osmotic stress. This suggests bacteria's ability in tolerating arsenic may also because of maintenance a homeostasis of intracellular ionic strength.

However, this result doesn't mean that the ability of arsenic-resistant bacteria in detoxifying arsenic is because of resistant to osmotic stress, nor expression of As-induced genes alleviated ionic osmotic stress. Indeed, cellular growth benefited from both arsenic stress and salt stress (Figure 3-6). As laboratory strain, *P. fluorescens* CHAO tolerated to -1.5M Pa of osmotic stress, but was not able to grow in the presence of arsenic.

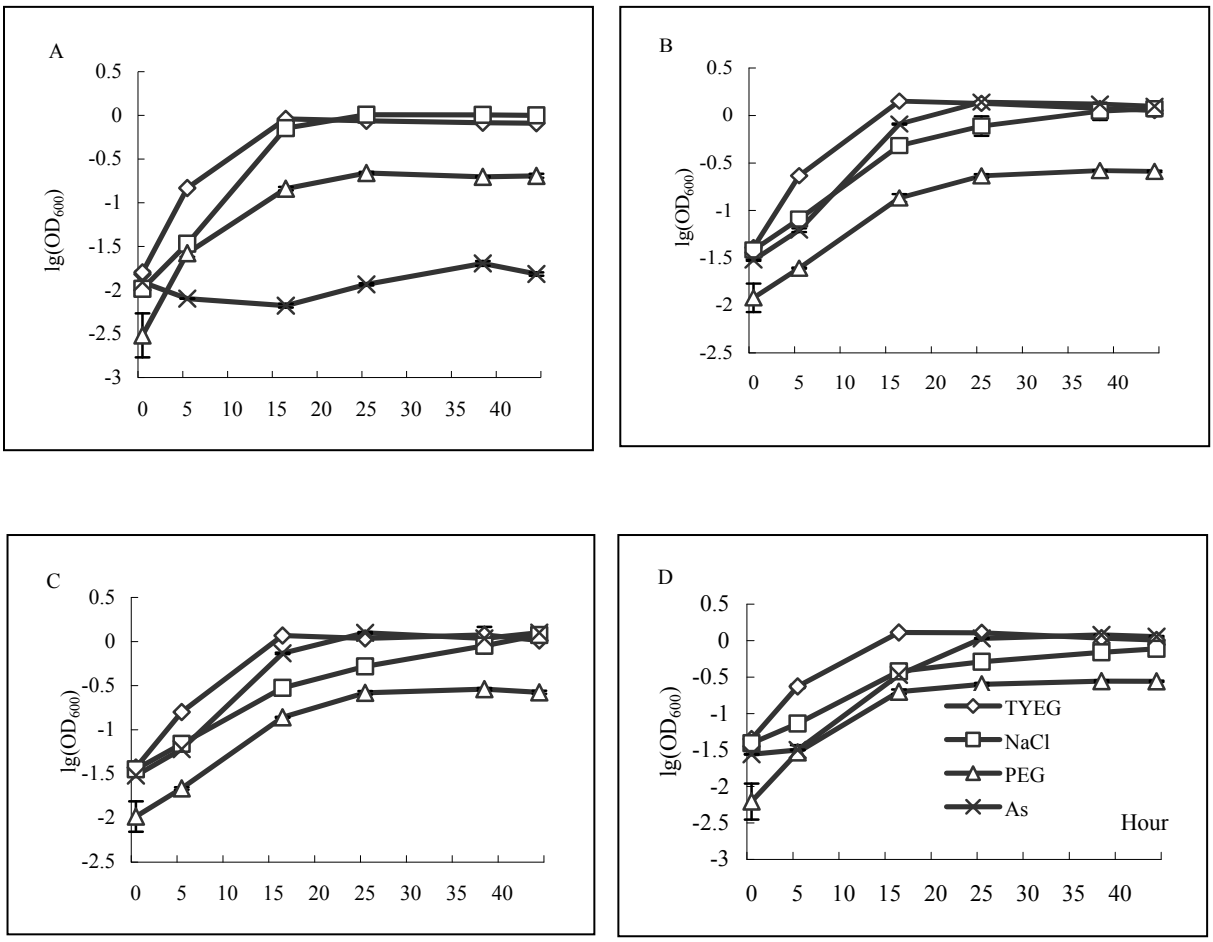


Figure 3-6. Bacterial growth under osmotic stress (-1.5MPa) generated by 400 mM of NaCl, 176 mM of sodium arsenate and 26% of PEG6000. The legend diamond denotes TYEG medium without additional osmotic source, rectangle denotes TYEG with NaCl, triangle denotes TYEG with PEG, and cross denotes TYEG medium with sodium arsenate. Pseudomonas fluorescens CHAO is a laboratory control strain. A: *Pseudomonas fluorescens* CHAO; B: *Naxibacter sp*. AH4; C: *Mesorhizobium sp.* AH5; D: *Methylobacterium sp*. AH6; E: *Enterobacter sp*. AH10; F: *Pseudomonas sp.* AH21; G: *Bacillus sp.* AH22; H: *Acinetobacter sp.* AH23; I: *Caryophanon sp.* AH28. Points are means and standard errors of 3 replicates.

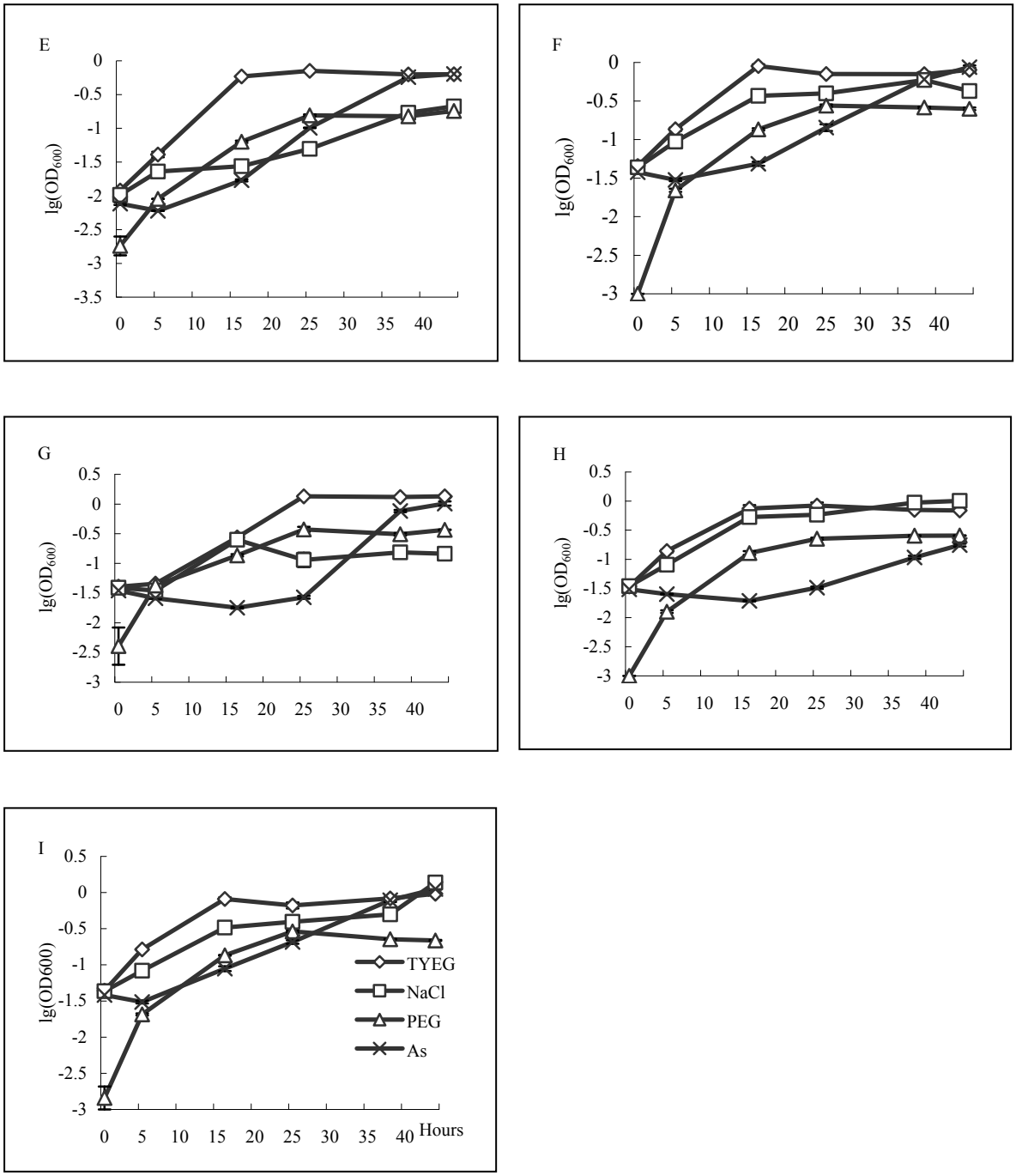


Figure 3-6. Continued.

3.5.3 Oxidative Stress Assay of Arsenic Resistant Bacteria

N,N'-Dimethyl-4,4'-bipyridinium dichloride (paraquat), one of the most widely used quaternary ammonium herbicides in the world, is extremely poisonous to organisms. It is easily reduced to a radical ion, which generates superoxide radical that reacts with unsaturated membrane lipids. Therefore, it is quick-acting, non-selective, and kills green plant tissue on contact conditions (Goel and Aggarwal, 2007).

It has been shown that inhibition of *Photorhabdus luminescens* growth on Petri dishes by 10 µl of 50 mM paraquat, and reduction of *P. luminescens* mRNA relative abundance in 50 min by 1 mM of paraquat in cell culture (Chalabaev et al., 2007). Figure 3-7 shows the result of paraquat growth inhibition of *Salmonella typhimurium* wild type and mutants. The mutants used are *S. typhimurium* SF1005, unmarked *rpoS* mutation, *S. typhimurium* cc1000 *rpoS*::*tet* (Tetracycline resistant gene) and *S. typhimurium* GS014 *oxyR*::Tn10 (Tn10 transposon). However, the mutants are not confirmed.

RpoS is a sigma factor, which is thought to be expressed only in stationary phase or cell starvation. However, it is also suggested that *rpoS* is induced by high cell density and that cells growth at these high densities seem to have undergone the general stress response, as judged by the production of trehalose (an osmoprotectant) and catalase (Mah and O'Toole, 2001). *OxyR* regulon is induced typically by hydrogen peroxide and tightly controls hydrogen peroxide level by increasing scavenging activities and limiting hydrogen peroxide generation in the respiratory chain (Gonzalez-Flecha and Demple, 2000).

The result in Figure 3-7 shows the inhibition zone around a glass disc containing 25 µl of 50 mM paraquat. The inhibition diameter reveals that *S. typhimurium* GS014 was more sensitive than wild type and *S. typhimurium* SF1005, suggesting reverse mutation in *S. typhimurium* SF1005 or synergistic reaction between paraquat and *rpoS* unmarked mutation. To confirm the result, bacterial growth under paraquat stress in 96 well culture plates was investigated.

Figure 3-8 shows the growth of *S. typhimurium* wild type and mutants in M9 minimal medium with 0.02% of glucose and 0.1, 0.3, 0.6, 0.9, 1.2, 1.5 or 2 mM of paraquat in 96-well cell culture plates at 37ºC. The 96 well plates can be easily contaminated because of strong evaporation at 37ºC, which explains the growth in 0.1 mM in Figure 3-8 A and 2 mM in Figure 3-8 B. Figure 3-8 B, C and D showed the increase of growth inhibition as paraquat concentration increased; however, paraquat had a smaller inhibition compared to relevant mutants. Among the mutants, *S. typhimurium* GS014 was more sensitive to paraquat than *S. typhimurium* cc1000 and *Salmonella typhimurium* SF1005. Both results confirmed reverse mutation in *S. typhimurium* SF1005 and *S. typhimurium* cc1000.

The fact that better growth in *S. typhimurium* mutants than the wild type was observed may result from synergistic reaction of paraquat and the *rpoS* mutations. Expressions of stress-related regulon regulated genes are quite energy consuming; however, in the mutants, low paraquat concentration may not be able to affect normal cellular proliferation. Structural alteration in reverse mutation may lead to insensitivity to paraquat induced oxidative stress. Besides, as a broad host pathogen, *S. typhimurium* had stronger tolerance to oxidative burst than *P. luminescens*; however, confirmation of synergistic effects is beyond this study. Therefore, *S. typhimurium* wild type and *S. typhimurium* GS014 were chosen as control to study oxidative stress growth of arsenic resistant bacteria. Because of the possible synergistic effect of paraquat and *oxyR* mutants, the following experiment was carried out with hydrogen peroxide. Compared to paraquat, hydrogen peroxide exerts internal oxidative stress, which is generated in cellular respiration under specific conditions, allowing this experiment to bypass the side effects of external reagents.

Arsenate at concentrations of 176 mM or 400 mM exerted great oxidative stress for bacteria, the 8 isolates belonging to 8 different genera were tested for anti-oxidative stress by measuring hydrogen peroxide inhibition zone with controls of *S. typhimurium* wild type and a

mutant having a Tn10 transposon inserted in *oxyR* gene. The result showed that, arsenic resistant bacteria were as sensitive to hydrogen peroxide as *S. typhimurium* wild type, except AH4, which was as sensitive as the *oxyR* mutant (Figure 3-9) (p-value <0.001). Bacterium AH4 was the only one that was able to grow in the presence of 2.4M NaCl (Figure 3-4).

Induced oxidative burst is a plant defense mechanism when encountering bacterial invasion (Bolwell and Wojtaszek, 1997). Meanwhile, in animals, phagocytic cells generate superoxide and other reactive oxygen species, which are involved in antibacterial activity (Hassett and Cohen, 1989). Usually pathogens are able to survive through those host defense systems. As a broad host of pathogen, *S. typhimurium* possess antioxidant defenses that allow its survival in inflammatory foci in either animal or plant hosts. The defenses include antioxidant enzymes such as superoxide dismutase and catalase, DNA repair systems, scavenging substrates, and competition with phagocytes for molecular oxygen. These defenses are regulated by global regulon such as OxyR in a coordinated fashion (Hassett and Cohen, 1989).

The fact that the arsenic resistant bacteria were as sensitive to hydrogen peroxide as *S. typhimurium* wild type indicates the strong ability of the bacteria in scavenging oxidative burst. This result suggests that the ability of arsenic-resistant bacteria in detoxifying arsenic was both because that expression of arsenic-induced genes alleviates cellular oxidative stress and the ability of the bacterial isolates in scavenging oxidative burst. The comprehensive effects were indicated by ARI in Figure 3-5.

Naxibacter sp. AH4 was as sensitive to hydrogen peroxide as *S. typhimurium* mutant. With such high sensitivity to hydrogen peroxide, AH4 needed to either sequestrate/immobilize all arsenic before it induced oxidative burst inside cells or prevent arsenic uptake, which is nearly impossible. This is because the chemical property of arsenic is similar to phosphorus, and arsenate is taken up nonspecifically by phosphate transporters

(Rosen, 2002). Therefore, lack of downstream arsenic detoxification ability in AH4 suggested a different arsenic detoxification mechanism in the isolate. In addition, AH4 was the only isolate that tolerated 2.4 M of NaCl (Figure 3-4), but not 26% of PEG (Figure 3-6). Further study is needed to unveil the specific genes that confer the unique detoxification ability to this strain.

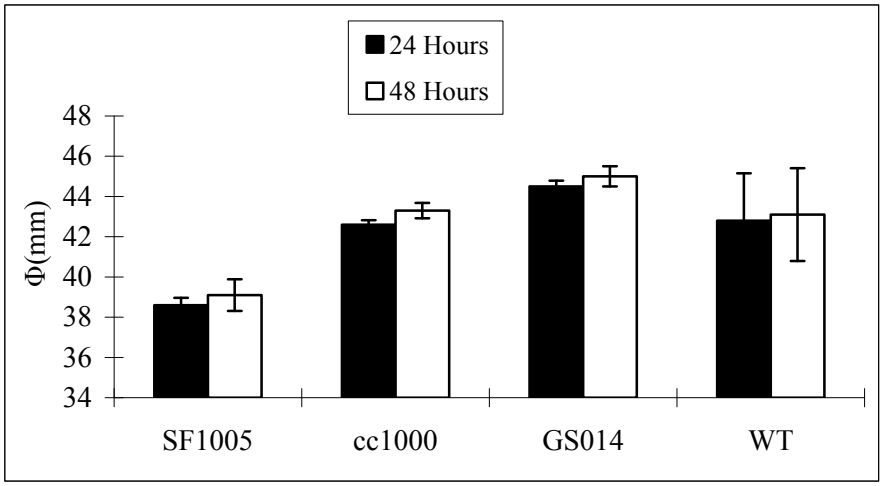


Figure 3-7. Oxidation inhibition area around a glass disc containing 25 µl of 50 mM N,N'-Dimethyl-4,4'-bipyridinium dichloride (paraquat) (α= 0.05); SF1005: *Salmonella typhimurium* SF1005 unmarked *rpoS* mutant; cc1000: *S. typhimurium* cc1000 *rpoS*:: *tet*; GS014: *S. typhimurium* GS014 *oxyR*:: Tn10; WT: *S. typhimurium* 14028 wild type. Y axis is diameter of the inhibition area on Petri dishes. Columns are means and standard errors of 3 replicates.

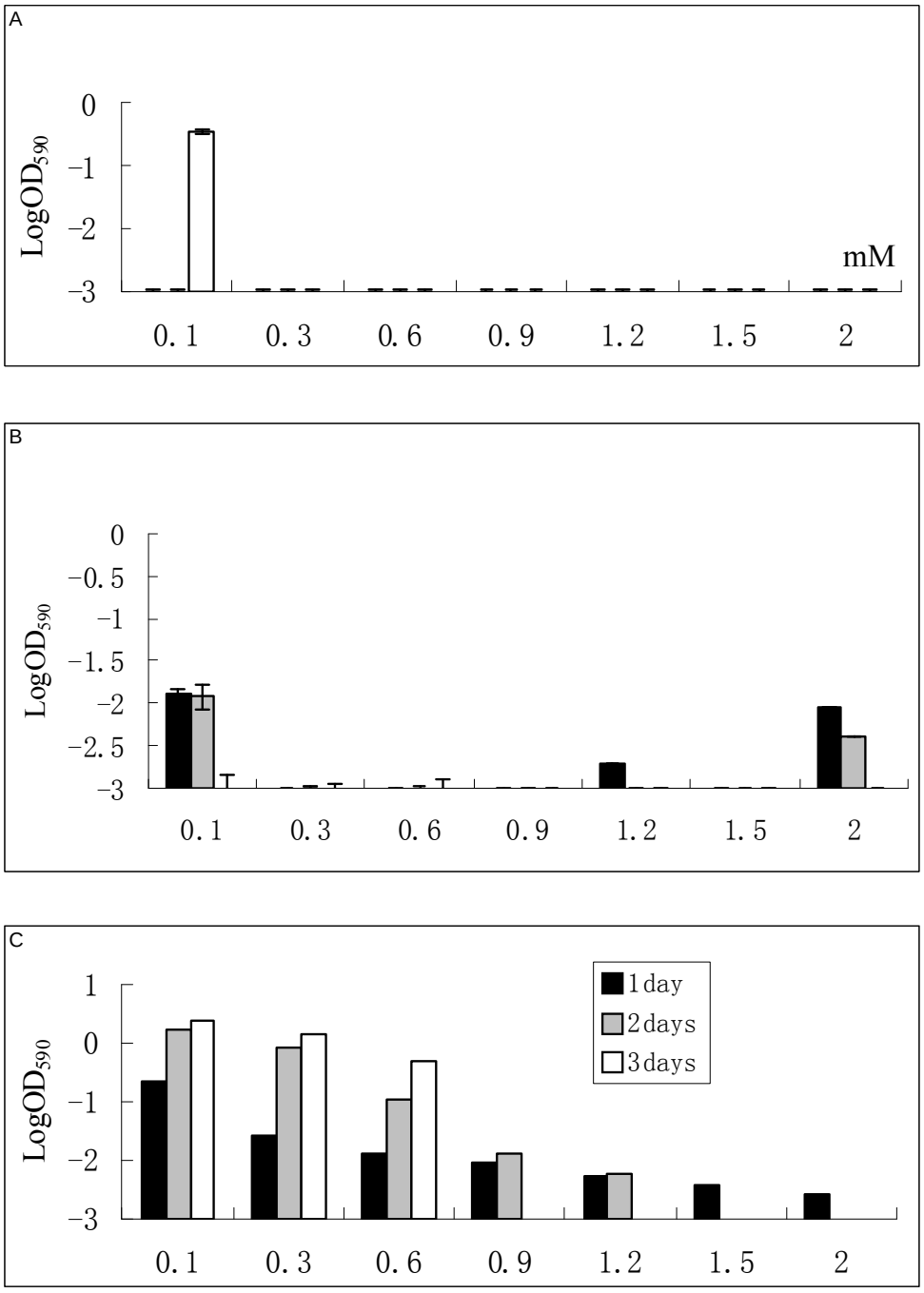


Figure 3-8. Growth of *S. typhimurium* wild type and mutants in M9 minimal medium with 0.02% of glucose and 0.1, 0.3, 0.6, 0.9, 1.2, 1.5 or 2mM of N,N'-Dimethyl-4,4'-bipyridinium dichloride (paraquat) in 96-well cell culture plates at 37 ºC; A: *Salmonella typhimurium* 14028 wild type; B: *S. typhimurium* GS014 *oxyR*:: Tn10; C: *S. typhimurium* cc1000 *rpoS*:: te; D: *S. typhimurium* SF1005 unmarked *rpoS* mutant. Columns are means and standard errors of 3 replicates.

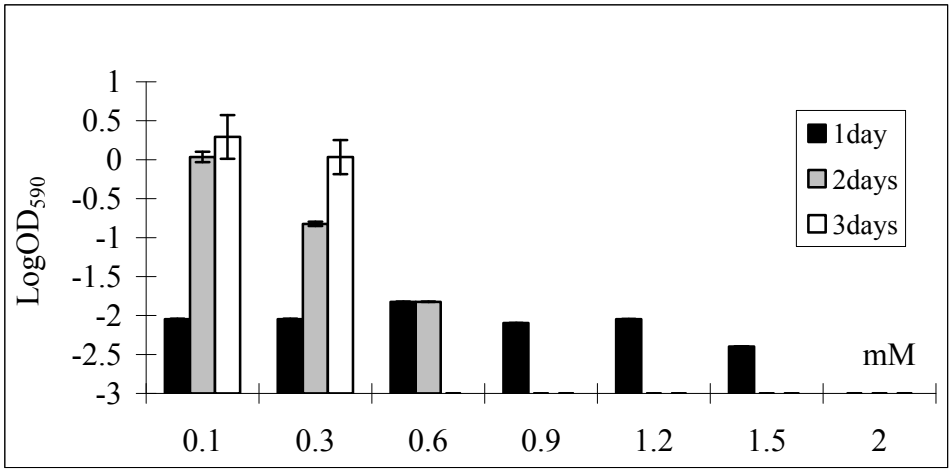


Figure 3-8. Continued.

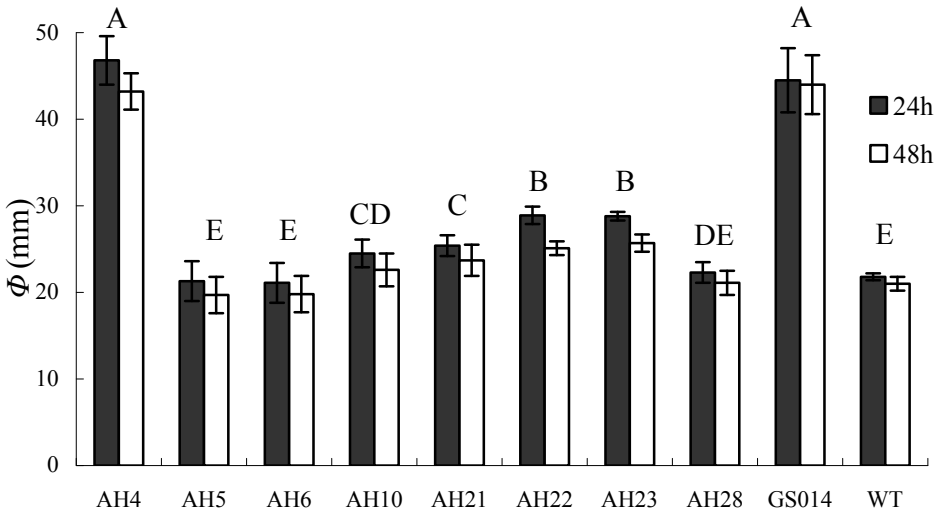


Figure 3-9. Oxidation inhibition zone around a glass disc containing 25 µl of 3% hydrogen peroxide (α= 0.05). AH4-AH28 are soil arsenic resistant isolates; GS014: *Salmonella typhimurium* LT2 *oxyR* :: Tn10; WT: *S. typhimurium* 14028 wild type. Y axis is diameter of the inhibition area on Petri dishes. Columns are means and standard errors of 3 replicates.

3.6 Arsenate Transformation by Arsenic Resistant Bacteria

3.6.1 *In vitro* Arsenic Transformation

It has been reported that majority of bacteria carry functional enzymes to transform arsenic, either reduce arsenate to arsenite or oxidize arsenite to arsenate. The arsenic resistant bacteria isolated from arsenic contaminated soils were tested for their ability in transforming arsenic during 32 hours of growth in TYEG medium, which was spiked with 375 µg of arsenate or arsenite. Figure 3-10-1 shows the speciation of arsenic in TYEG medium without bacterial inoculation, which confirms that both arsenate and arsenite were stable in TYEG medium with agitation. Figure 3-10-5 shows the speciation of arsenic in medium with a laboratory strain *Sinorhizobium sp.* MG32, Figure 3-10-2, 3, 4 show the high efficiency of the soils isolates *Naxibacter sp.* AH4, *Mesorhizobium sp.* AH5, *Pseudomonas sp.* AH21in reducing arsenate to arsenite. All arsenate was reduced to arsenite after 32 hours, with the half life of arsenate in presence of As resistant bacteria being 22 hours for AH4, 18 hours for AH5 and 12 hours for AH21 in this study.

Figure 3-11 shows the final OD at 600 nm after 32 hours of growth in the presence of AsV or AsIII. MG32 growth was inhibited by 1 mM of arsenite, though it was resistant to 1 mM of arsenate. In comparison, arsenic-resistant bacteria *Naxibacter sp.* AH4, *Mesorhizobium sp.* AH5, *Pseudomonas sp.* AH21 tolerated both arsenate and arsenite. While there was neither oxidation nor reduction happened in the medium with MG32, the arsenic resistant bacteria reduced arsenate to arsenite efficiently (Figure 3-10). There was no arsenate detected after 32 hours of growth in the medium started with 1 mM of sodium arsenate (Fig. 3-10).

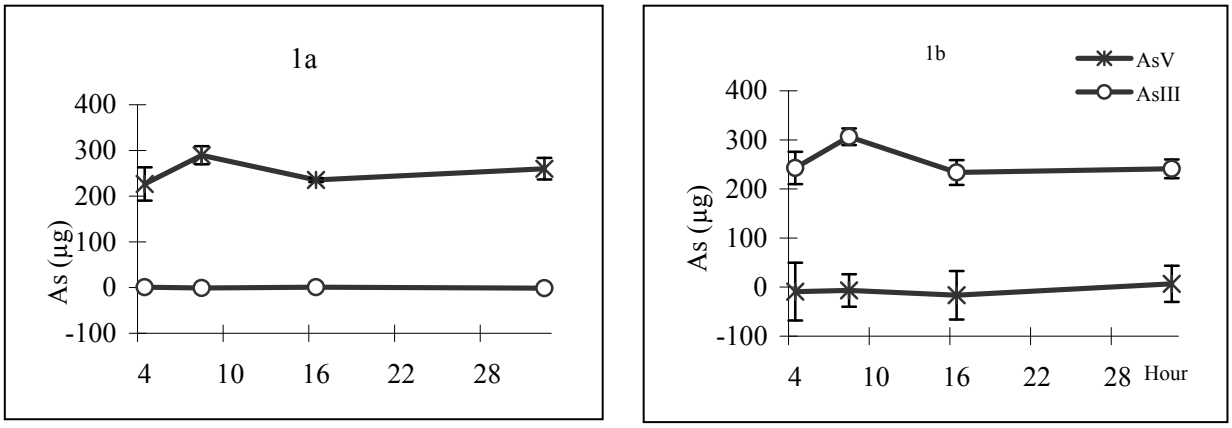


Figure 3-10. Arsenic transformation by arsenic-resistant bacteria AH4, AH5 and AH21 during 32 h of growth in TYEG medium spiked with 375 µg of arsenate (AsV) or arsenite (AsIII). A laboratory strain *Rhizobium sp.* MG32 served as control. 1: no bacterium; 2: *Naxibacter sp.* AH4; 3: *Mesorhizobium sp.* AH5; 4: *Pseudomonas sp.* AH21; 5: *Sinorhizobium sp.* MG32. a: started with AsV; b: started with AsIII. Points are means and standard errors of 3 replicates.

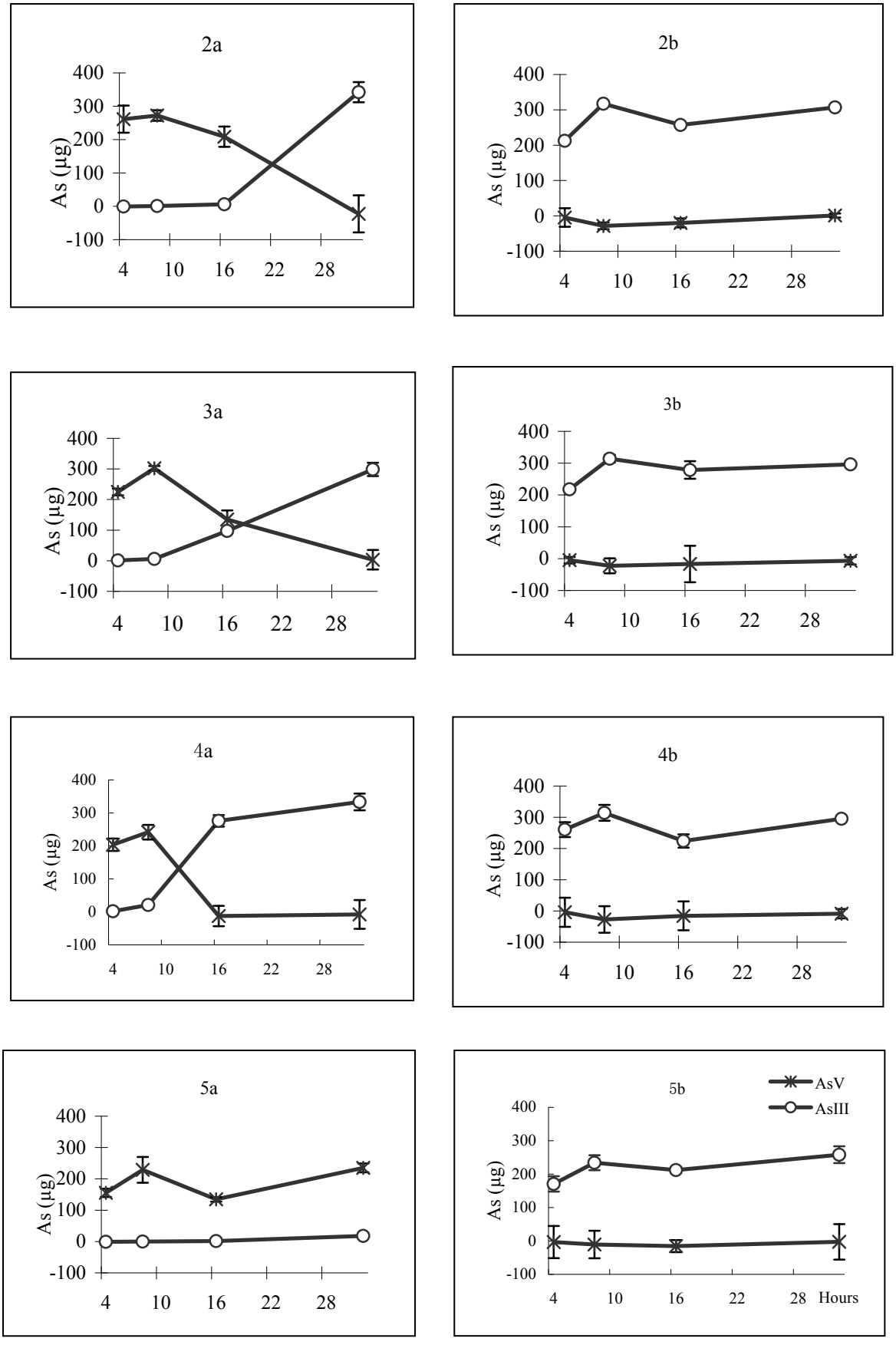


Figure 3-10 Continued.

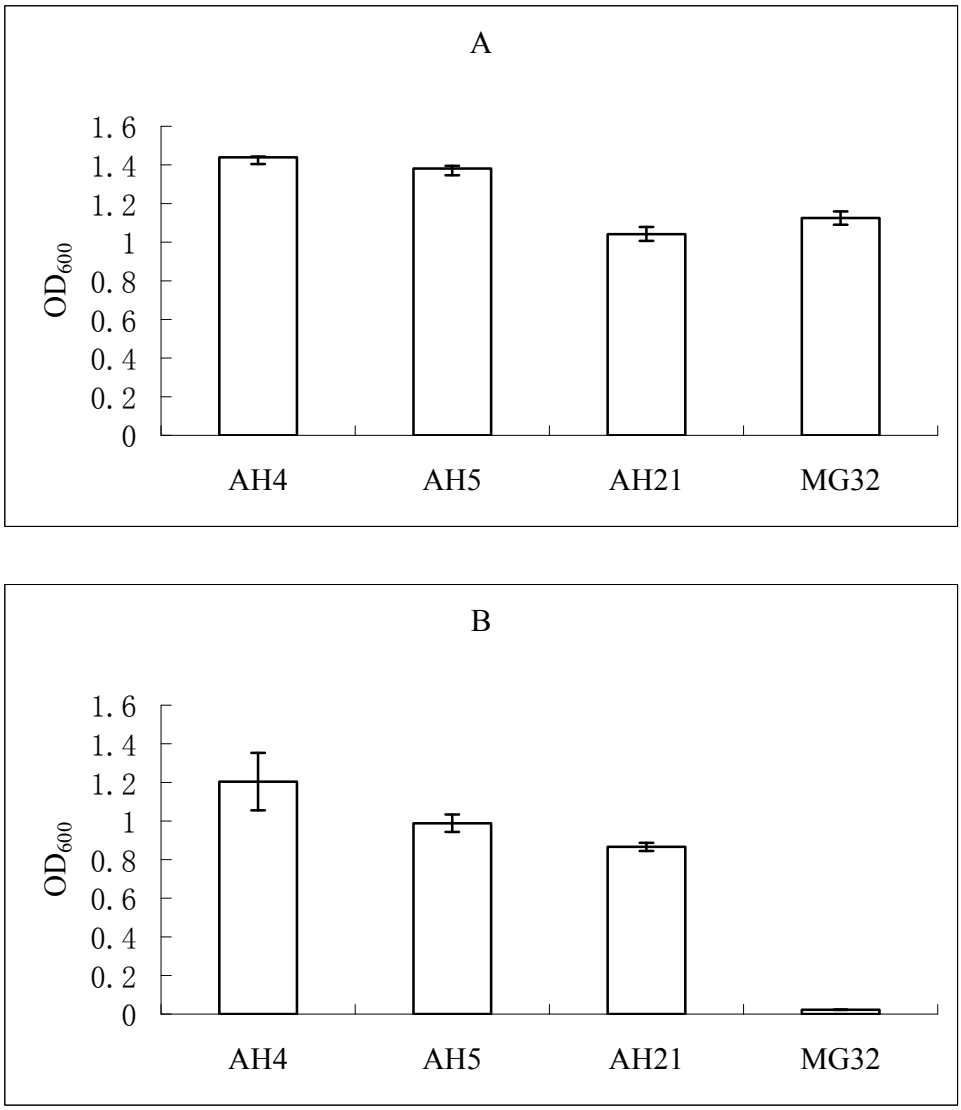


Figure 3-11. Growth of arsenic-resistant bacteria AH4, AH5 and AH21 after 32 h in TYEG medium spiked with 375 μg of arsenate (A) or arsenite (B). A laboratory strain *Rhizobium sp.* MG32 served as control. Points are means and standard errors of 3 replicates.

3.6.2 Reductase Enzyme Activity Assay and Functional Gene Searching

While the arsenic transformation experiment showed efficient reduction by arsenic resistant bacteria isolated from arsenic contaminated soils, this experiment tested enzymatic arsenate reduction, specifically the glutaredoxin (grx, specifically, *E. coli* grx2) dependent arsenate reductase activity.

During arsenic reduction, glutaredoxin provides electron to arsenate, and the oxidized form of glutaredoxin is then reduced by GSH. Finally oxidized GSSG is reduced again by yeast glutathione reductase, which uses NADPH as electron donor and results in the decrease

of absorption. Figure 3-12 A and B are background control, which showed a very small arsenate reduction by *E. coli* glutaredoxin, confirming that glutaredoxin is able to reduce arsenate slowly (Shi et al., 1999). Figure 3-12 C, D, E are enzymatic reduction by total protein from *E. coli* DH5α, *Pseudomonas sp.* AH25 and *Pseudomonas sp.* AH45. The total protein concentrations were 13.5, 94.5 and 203 μg, respectively.

Chemical transformation results and enzymatic assay together prove the existence of a specific gene system to transform and detoxify arsenic by arsenic resistant bacteria. Although the isolation method used in this study recovered aerobic heterotrophic bacteria from the rhizosphere of the arsenic hyperaccumulator *P. vittata,* the isolates could be also facultative anaerobics; therefore, both arsenic specific resistant mechanisms were studied. Several pairs of primers had been tried to clone *ars* operon genes or *arrA* gene. Those primers were either reported in previous studies or designed based on *ars* operon sequence of *P. aeruginosae* PAO1. All positive controls were sequenced to confirm if the primers were working.

There was no positive PCR product amplified by the primers based on *arsR*, *arsB* and *arsC* genes in *Pseudomonas aeruginosae* PAO1 in tested arsenic resistant bacteria. Southern hybridization with *arsC* probe from PAO1 also showed a negative result (Figure 3-13). Figure 3-13 A shows DNA in agarose gel and Figure 3-13 B shows hybridization results in nylon membrane.

The lanes in the upper half are *arsC* PCR products from the genomic DNA templates of *Naxibacter sp.* AH4, *Mesorhizobium sp.* AH5, *Methylobacterium sp. Enterobacter sp.* AH10*, Pseudomonas sp.* AH21, *Bacillus sp.* AH22*, Acinetobacter sp.* AH23, *Pseudomonas sp.* AH25, *Caryophanon sp.* AH28, *Pseudomonas sp.* AH33, *Pseudomonas sp.* AH34, *Pseudomonas sp.* AH45, and *P. aeruginosae* PAO1, respectively, with primers based on PAO1 *arsC* gene. The probe was confirmed by sequencing.

The lanes in the lower half are EcoRI partial digested genomic DNA from *Naxibacter sp.* AH4, *Mesorhizobium sp.* AH5, *Methylobacterium sp. Enterobacter sp.* AH10*, Pseudomonas sp.* AH21, *Bacillus sp.* AH22*, Acinetobacter sp.* AH23, *Pseudomonas sp.* AH25, *Caryophanon sp.* AH28, *Pseudomonas sp.* AH33, *Pseudomonas sp.* AH34, *Pseudomonas sp.* AH45, PCR2.1 with *arsC* insertion (uncut), and *Pseudomonas aeruginosae* PAO1.

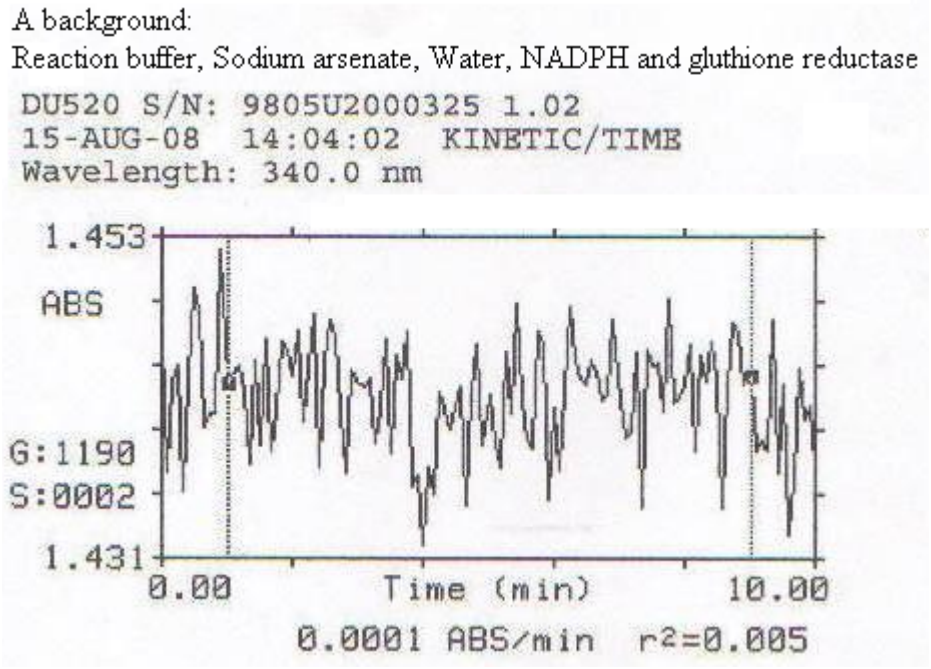
While there was strong signal in the positive controls, there was no hybridization between genomic DNAs and *arsC* from *P. aeruginosae* PAO1, which may because of low sensitivity of DIG DNA labeling method and low copy of *arsC* from the arsenic resistant bacteria, or because there was no similar *arsC* as *P. aeruginosae* PAO1 in arsenic resistant bacteria. If so, the strong hybridization signals between PCR products resulted from the primer sequence hybridizations. Phylogenetic analysis of bacterial and archaeal *arsC* gene sequences has shown that *arsC* phylogeny is complex and is likely the result of a number of evolutionary mechanisms, in which inconsistencies between *arsC* and 16S rRNA are apparent for some taxa, and other isolated taxa possess *arsC* genes that would not be expected based on known evolutionary relationships (Jackson and Dugas, 2003). Therefore, the experiment result suggests novel functional arsenate reductase genes or very low similarity between different strains, resulting in the difficulties to locate the target gene.

ArrA gene amplification was positive based on the arsenate respiration marker designed by D. Malasarn (Malasarn et al., 2004). The PCR result is shown in Figure 3-14. Positive control was confirmed by sequencing. However, except partial sequences of *arrA* gene were found in some of the isolates such as *Pseudomonas sp.* AH25 and *Pseudomonas sp.* AH45, all other clones showed novel sequences, which had never been reported and there was no similar sequences available in GenBank. Blast result of *Pseudomonas sp.* AH25, which has the highest similarity among those sequenced soil isolates PCR products, is shown in Figure

3-14 b. Not only there was low similarity between soil isolates *arrA* genes and *Shewanella sp. arrA*, but also, upon the reported *arrA* genes, there was only a fragment of 103 nucleotides similarity between *Shewanella sp. arrA* and *Bacillus selenitireducens* strain MLS10 *arrA* gene (Genbank accession no. AY283639). The cloning results is the conserve region of the 2.5k base pairs *arrA* gene according to D. Malasarn (Malasarn et al., 2004). However, to further confirm the existence of the *arr* operon, it is required to either test bacterial enzymatic arsenate reduction in strict anaerobic condition or employing better cloning strategy.

Though arsenic resistant plasmid determination was negative, there is no guarantee that the plasmid functional genes will confer arsenic resistance to *E. coli* DH5α, which may cause false negative results. This can be caused by the difference between plasmid hosts, such as copy number restriction and gene transcription regulation. In addition, different arsenate reductase genes require different cofactors. Arsenate reductase in *Pseudomonas* species may not favor glutaredoxin in *E. coli* DH5α. Moreover, some of the plasmid encoding *ars* operon genes are huge and won't be able to be recovered by the plasmid extraction method used here. In this case, those plasmids are considered as genomic DNA while we were searching for functional genes. A genomic DNA library was also tried to search for functional genes. However, due to the fact that different arsenate reductase genes require different cofactors and other host differences, there is no functional gene found so far.

A background:
Reaction buffer, Sodium arsenate, Water, NADPH and gluthione reductase



B Background added with *E. coli* glutaredoxin

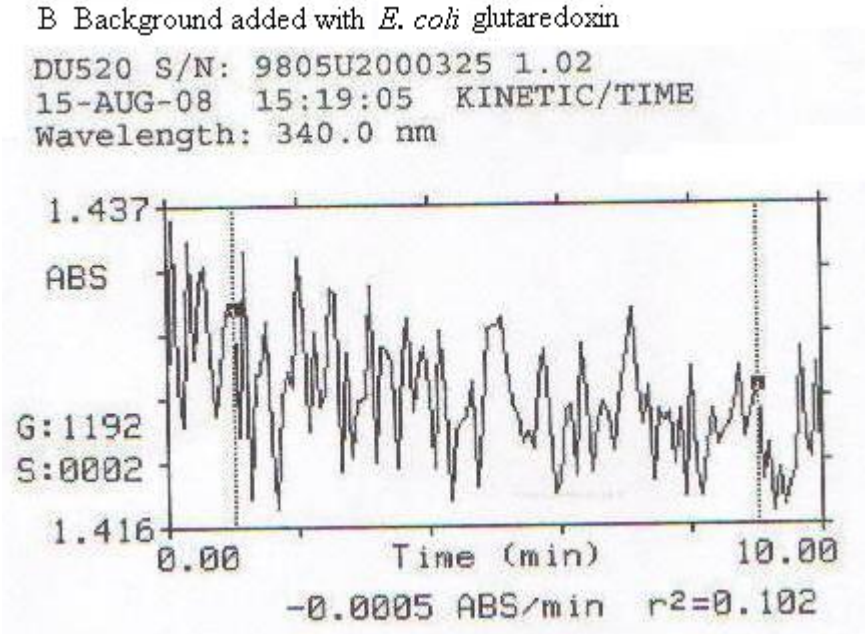
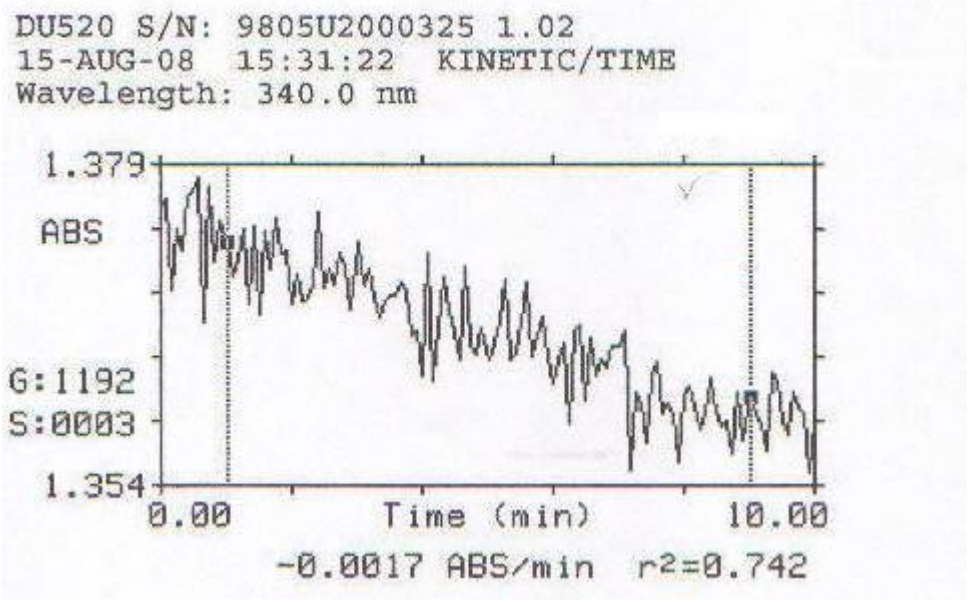


Figure 3-12. Arsenate reductase assay of arsenic resistant bacteria isolated from arsenic contaminated soils by measuring NADPH absorption at 340 nm. This method is to analysis the enzymatic activity of the glutaredoxin (grx) dependent arsenate reductase activity. Reaction solution in 2mL cuvette contains 150 µl bacterial protein extracts, 1 ml reaction buffer, 10 µM sodium arsenate, 0.5 mM NADPH, 1 mM GSH, 2U yeast glutathione reductase and 0.02µM *E. coli* Glutaredoxin 2. A and B are background control, which shows a very small arsenate reduction by *E. coli* glutaredoxin. C, D, E are enzymatic reduction by total protein from *E. coli* DH5α, *Pseudomonas sp.* AH25 and *Pseudomonas sp.* AH45. The total protein concentrations were 13.5, 94.5 and 203 µg, respectively.

C Background, *E. coli* glutaredoxin and *E. coli* DH5α total protein extracts



D Background, *E. coli* glutaredoxin and total protein extracts from *Pseudomonas sp.* AH25

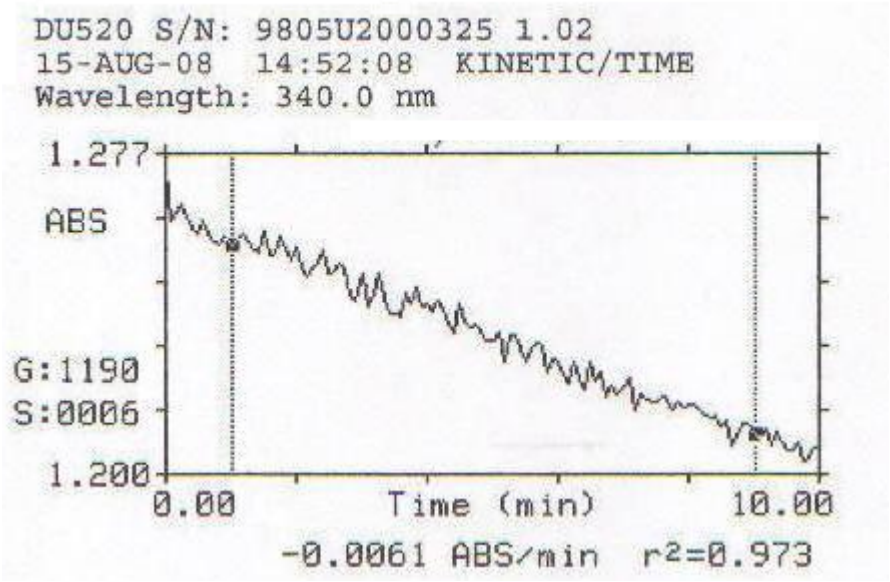


Figure 3-12. Continued.

E Background, *E. coli* glutaredoxin and total protein extracts from *Pseudomonas sp.* AH 45

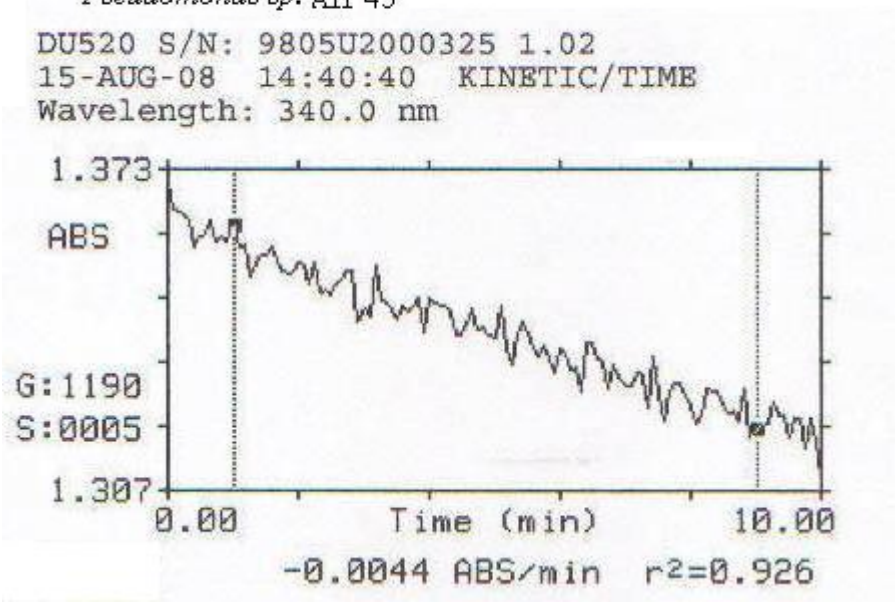


Figure 3-12. Continued.

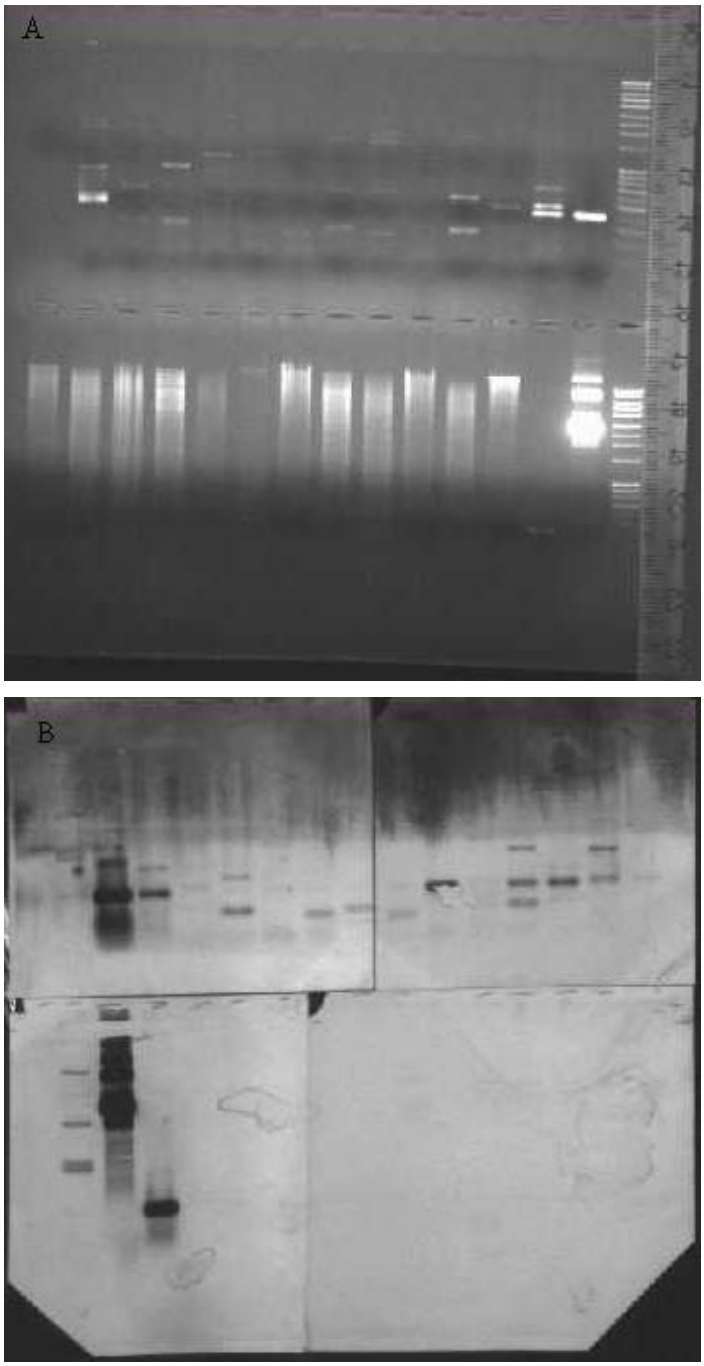


Figure 3-13. *arsC* gene Southern Hybridization. A shows DNA in agarose gel and B shows hybridization results in nylon membrane. The lanes in the upper half are *arsC* PCR products from the genomic DNA templates of *Naxibacter sp.* AH4, *Mesorhizobium sp.* AH5, *Methylobacterium sp.* AH6, *Enterobacter sp.* AH10, *Pseudomonas sp.* AH21, *Bacillus sp.* AH22, *Acinetobacter sp.* AH23, *Pseudomonas sp.* AH25, *Bacillus sp.* AH28, *Pseudomonas sp.* AH33, *Pseudomonas sp.* AH34, *Pseudomonas sp.* AH45, and *Pseudomonas aeruginosae* PAO1, respectively. The lanes in the lower half are EcoRI partial digested genomic DNA from *Naxibacter sp.* AH4, *Mesorhizobium sp.* AH5, *Methylobacterium sp.* AH6, *Enterobacter sp.* AH10, *Pseudomonas sp.* AH21, *Bacillus sp.* AH22, *Acinetobacter sp.* AH23, *Pseudomonas sp.* AH25, *Bacillus sp.* AH28, *Pseudomonas sp.* AH33, *Pseudomonas sp.* AH34, *Pseudomonas sp.* AH45, PCR2.1 with *arsC* insertion (uncut), and *Pseudomonas aeruginosae* PAO1.

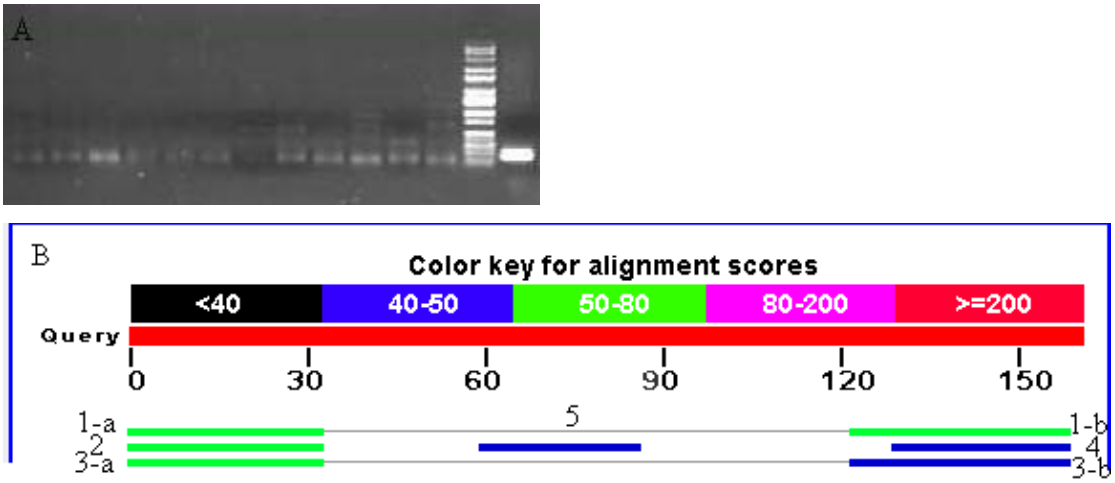


Figure 3-14. *arrA* gene cloning. A: PCR products in agarose gel. Lanes are PCR product from genomic DNA templates of *Naxibacter sp.* AH4, *Mesorhizobium sp.* AH5, *Methylobacterium sp.* AH6, *Enterobacter sp.* AH10, *Pseudomonas sp.* AH21, *Bacillus sp.* AH22, *Acinetobacter sp.* AH23, *Pseudomonas sp.* AH25, *Bacillus sp.* AH28, *Pseudomonas sp.* AH34, *Pseudomonas sp.* AH43, *Pseudomonas sp.* AH45, and *Shewanella sp.* ANA-3, respectively. *Shewanella sp.* ANA-3 *arrA* PCR product was confirmed by sequencing. B: BLAST result of *Pseudomonas sp.* AH25 *arrA* gene. Left side sequences are identical with *arrA* forward primer. No similarity between right side sequences and *arrA* primers. Similar BLAST results in AH34 and AH45 (data not show, sequences available in Appendix C), which suggested novel *arrA* genes. Sequence in B: 1. HRD9 arsenate respiratory reductase (*arrA*) gene. GenBank accession No. AY707754.1 a: Identities = 33/33 (100%), Gaps = 0/33 (0%); b: Identities = 35/38 (92%), Gaps = 1/38 (2%). 2. GL-ARRA1 arsenate respiratory reductase-like (*arrA*) gene. GenBank access No.EF014944.1. Identities = 32/33 (96%), Gaps = 0/33 (0%). 3. HRR23 arsenate respiratory reductase (*arrA*) gene. GenBank access No.AY707770.1. a: Identities = 32/33 (96%), Gaps = 0/33 (0%); b: Identities = 32/33 (96%), Gaps = 0/33 (0%). 4. HRR20 arsenate respiratory reductase (*arrA*) gene. GenBank accession No. AY707768.1, Identities = 28/30 (93%), Gaps = 0/30 (0%). 5. Homo sapiens cDNA, GenBank access No AK307664.1, Identities = 25/27 (92%), Gaps = 0/27 (0%).

This study isolated a group of bacteria from arsenic contaminated soils where *P. vittata* grew. This included phosphorus and arsenic solubilizing bacteria, which can potentially enhance phytoremediation through improving metal removal rate and increasing harvested biomass. In addition, we have identified and purified the most arsenic resistant bacteria, which were able to tolerate up to 30,000mg/kg of arsenic in liquid culture, the highest level reported to date.

Most of studies on arsenic resistant bacteria have focused on specific arsenic resistant mechanisms, i.e., functional genes in detoxification mechanisms through transformation and sequestration of arsenic species in either vacuoles (if the organisms have) or outer membrane; However, little information is available at global metabolic levels such as the osmotic stress between membranes when arsenic are stored/accumulated, oxidative stress generated during the exposure, and the impacts of those stresses in cellular growth.

In this study, for the first time, multiple types of arsenic resistance were proposed. Experiment results support that high arsenic resistant ability of arsenic resistant bacteria was due to not only their efficiency in transformation and sequestration arsenic, but also their ability in scavenging oxidative burst and counteracting with different osmotic stresses. In other words, bacteria growth benefited from both arsenic induced genes that alleviate arsenic toxicity and from general high level of cell metabolisms in counteracting cellular growth stresses. Arsenic resistant index (ARI) was developed to qualify the two level of arsenic detoxification. In the index, ARI indicated the comprehensive effects of bacteria's ability in reducing and extruding arsenic besides bacteria's ability in overcoming toxicity at cellular level.

APPENDIX A
TWELVE MOST ARSENIC RESISTANT ISOLATES16S RIBOSOMAL RNA GENE SEQUENCES

Bacteria are denoted by '>' follows with strain names.

>AH1
ggcatgctttacacatgcaagtcgaacggcagcacgggcttcggcctggtggcgagtggcgaacgggtgagtaatacatcggaacgtacccagaa
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tctgattagctagttggtggggtaaaggctcaccaaggcgacgatcagtagctggtctgagaggacgaccagccacactggaactgagacacggtc
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>AH4
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>AH5
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>AH6
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>AH10
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APPENDIX B
REFERENCES FOR 16S RIBOSOMAL RNA GENE SEQUENCES

References 16s rDNA sequences in Figure 3-2 and corresponding GenBank accession numbers.

Bacteria are denoted by '>' follows with GenBank accession number, genus name, family name and strain name if it has one. Only informatic nucleotides using in constructing phylogenetic tree showed.

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>FM179768 *Acetobacter pasteurianus* AUC25
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>AF181576 *Pseudomonas cf. monteilii*
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>AJ006086 *Bacillus silvestris*
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>EU194334 *Pseudomonas plecoglossicida* XJUHX-16
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>AF368755 *Pseudomonas saccharophila*
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>EU239475 *Pseudomonas plecoglossicida* XJUHX-15
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>EU239464 *Pseudomonas putida* XJUHX-1
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>EF178450 *Pseudomonas entomophila* 2P25
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>EU857417 *Pseudomonas taiwanensis* BF-S2
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APPENDIX C
ARSENATE REDUCTASE GENE SEQUENCES

Sequencing results of arsenate reductase genes, partical sequences.

>*Shewanella sp.* ANA-3 *arrA*

```
   1 aaagtaacgc tctatagggc gaattgggcc ctctagagca tgctcgagcg
  51 gccgccagtg tgatggatat ctgcagaatt cggcttaagg tgtatggaat
 101 aaagcgtttg tgggtgactt tattgagggt aaaaacctgt ttaaagcagg
 151 taaaaccgtc agtgtcgaga gctttaaaga aacccatacc tacggtttag
 201 tcgaatggtg gaaccaggcc cttaaagatt acactcctga atgggcacct
 251 gaaatcacag gaagccgaat tccagcacac tggcggccgt tactagtgga
 301 tccgagctcg gtaccaagct tggcgtaatc atggtcatag ctgtttcctg
 351 tgtgaaattg ttatccgctc acaattccac acaacatacg agccggaagc
 401 ataaagtgta aagcctgggg tgcctaatga gtgagctaac tcacattaat
 451 tgcgttgcgc tcactgcccg ctttccagtc gggaaacctg tcgtgccagc
 501 tgcattaatg aatcggccaa cgcgcgggga gaggcggttt gcgtattggg
```

>AH25 *arrA*

```
   1 aattgatcga ctagtatagg gcgaattggg ccctctagat gcatgctcga
  51 gcggccgcca gtgtgatgga tatctgcaga attcggctta aggtgtatgg
 101 aataaagcgt ttgtgggcga tttgctttcc gaggcagtca tctgtggctt
 151 cactactttg tcttcacagg ctgtggtcgc tcactgtcaa cagcaaatgt
 201 gtgtcaggac aaaatattaa cccctaaatg ggcacctgaa atcacaggaa
 251 gccgaattcc agcacactgg cggccgttac tagtggatcc gagctcggta
 301 ccaagcttgg cgtaatcatg gtcatagctg tttcctgtgt gaaattgtta
 351 tccgctcaca attccacaca acatacgagc cggaagcata aagtgtaaag
 401 cctggggtgc ctaatgagtg agctaactca cattaattgc gttgcgctca
 451 ctgcccgctt tccagtcggg aaacctgtcg tgccagctgc attaatgaat
```

>PAO1*arsC*

```
   1 cggtgaacga ctctataggg cgaattgggc cctctagatg catgctcgag
  51 cggccgccag tgtgatggat atctgcagaa ttcgcccttc tgttcatgtg
 101 cacggccaac ggccgctggc tttcatggcg ataggtgata gcatagagcc
 151 atcattcgca gcgacgctct gccgcgaaga ccacgaaaga agcgcgtcat
 201 gatgaccgag cacgatgacc cgaccctgga ccgcctgaag caccacttcg
 251 cccagcgagt gatcaaccag gcgcgccagg ttctggaggt ctggcaacgc
 301 ctgacccgcg cggagtggaa cagcgacggc atggaagaac tggccgacgc
 351 caccctgcgc ctgcagcgct acgccgaacg cttcgagcaa gccgagcatg
 401 cccagttggc cgtgcacatg aacagaaggg cgaattccag cacactggcg
 451 gccgttacta gtggatccga gctcggtacc aagcttggcg taatcatggt
 501 catagctgtt tcctgtgtga aattgttatc cgctcacaat tccacacaac
 551 atacgagccg gaagcataaa gtgtaaagcc tggggtgcct aatgagtgag
 601 ctaactcaca ttaattgcgt tgcgctcact gcccgctttc cagtcgggaa
 651 acctgtcgtg ccagctgcat taatgaatcg gccaacgcgc ggggagaggc
 701 ggtttgcgta ttgggcgctc ttccgcttcc tcgctcactg actcgctgcg
 751 ctcggtcgtt cggctgcggc gagcggtatc agctcactca aaggcggtaa
 801 tacggttatc cacagaatca ggggataacg caggaaagaa catgtgagca
 851 aaaggccagc aaaaggccag gaaccgtaaa aaggcccgcg ttgctggcgt
 901 ttttccatag gctccgcccc ccctgacgag catcacaaaa tcgacgctca
 951 gtcagaggtg gcgaacccga caggactata aagataccag gcgttccccc
1001 tggaagcctc cctcgtgcgc tctctgttcg gacctgccgc ttaccggata
1051 cctgtcccgc cttctcctcg ggaagcggtg gcggcttctc atagctcacg
1101 ctgtaggtat tctcaagttc ggggtagttc ggtcgctcca aagcctgctg
1151 ggtgcgaccc cgttcgagcc cggacgcttg cgccttatat ccggtgta
```

>AH4 *arsC*

```
   1 cacgtgtatc gactactata gggcgaattg ggccctctag atgcatgctc
  51 gagcggccgc cagtgtgatg gatatctgca gaattcgccc ttgcattctt
```

101 tccgaagcca tgttcaagta cacgtcccac gctggcgagg gccacaccat
151 cgctgaaatg gacgatgaaa caaagaagga actgtggcag aagaagctgc
201 ctcgactccc cggccatcgc agcgagctcg acaaagtcaa gatcgagaag
251 gtcaagcccg acaagaagga agcgcttctt cgagtcgagg actacattca
301 caccaaggcc aatcatacga ctttcacgga ggccctcacc atttgcccgt
351 tcgaccttct cgagaacgac gccttgaaga ccgaaatggt ccgtggcgga
401 atacagcttt tcggttccct cccgacggcg caagacgccc aagagaagct
451 agacgcgctt gagagcgtct ttcctcagtg gaacgccatc gcgaccagct
501 caagcgtgag ctaagggcga

>AH34 *arsC*
1 acggagtatc gactctatag ggcgaattgg gccctctaga tgcatgctcc
51 gagcggccgc cagtgtgatg gatatctgca gaattcgccc ttgcattctt
101 tccgaagcca tgttcaaggc cgcctacgga gaatctccat acaactacct
151 catgacacgg cgcatcgagc gggccatggc cctgctccgc gcgggaacca
201 gcgtcaccga tgcctgcatg gaagtcggct gtacctcgtt gggctcgttc
251 agcacgcgtt tcaccgaaat cgtgggaatc aaccccagcg agtaccgcgc
301 ccgggagcac cacgctgtga aggccatgcc caactgcatc gcgaccagct

>AH45 *arsC*
1 ctaacgtgaa acgactccta tagggcgaat tgggccctct agatgcatgc
51 tcgagcggcc gccagtgtga tggatatctg cagaattcgc ccttgtattc
101 tttccgaagc catgttcaag ttcagggcct gcaggggcgg caacccgggg
151 gtgtagcgcc gggcgatcac ttcatcgcgc ggctgttggt cgccatggaa
201 gaacaggcac gcggaaagcc cgaggcaacg cgaacggaat tcgccgatgt
251 agtacgcctt gcctgcttcc aggtgcatgg gcacaagaaa ctgttcccgt
301 gcccggatgg agctgtagcc accgttgtag ccgttccaga agaactggaa
351 gtcatagaac tcgtagtcgc cgggcttgag gggcatgacg aacacgctgg
401 cctcgccggg cagcgcctgc ttgtcggggc cacgcacctc ttcgatgtcc
451 ttcggggtgt gggccacttc ggcgaacccc caccatgccg ccgcgccatc
501 ctgcgcgcca cgtttgcgca ggaggatgcg ttggttggtg taggcactat
551 cgcgaccagc tcaagcgtga gct

APPENDIX D
ARSB GENE SEQUENCES

>AH23 *arsB*

```
  1 acagaagatc cgctactata gggcgaattg ggccctctag atgcatgctc
 51 gagcggccgc cagtgtgatg gatatctgca gaattcggct tgcggaaata
101 gaggaacagc accacgggca ggacagcgtg gacgtcctcg acggcaacga
151 tggggaaccc gatgatctgg atcttgtccg tacgcgtgaa gtggccgacg
201 cgcaaacggt gcatgccgat ggcacgcccg acgtaatgcc gcttccagtt
251 ttcggtggct tcggacgggt tgccggggtt ttccagccag tcgatcttgc
301 cgccttcggg gtcagcgtgg tggatggttc cgcccggacc ataaagctgg
351 tagcaaacca ccaggtccgt ggtgccgtca ccagtgatgt caccgaagtc
401 catgccgacg ggttctttga tcttgtccag caccagcttc ttttcccagg
451 tggggttttt gtaccagtag atttcaccga ctttcaggcc gtagccaacg
```

>AH25 *arsB*

```
accagtatcgactactatagggcgaattgggccctctagatgcatgctcgagcggccgccagtgtgatggatatctgcagaattcggcttgcggaaatagaggaacagc
accacgggcaggacagcgtggacgtcctcgacggcaacgatggggaacccgatgatctggatcttgtccgtacgcgtgaagtggccgacgcgcaaacggtgcatgc
caatggcacgcccgacgtaatgccgcttccagttttcggtggcttcggacgggttgccggggttttccagccagtcgatcttgccgccttcggggtcagcgtggtggatg
gttccgcccggaccataaagctggtagcaaaccaccaggtccgtggtgccgtcaccggtgatgtcaccgaagtccatgccgacgggttctttgatcttgtccagcaccag
cttcttttcccaggtggggtttttgtaccagtagatttcaccgactttcaggccgtagccaacgaggtccggcttgccgtcgccgtcaatatctgccgcttccagccagtagc
cgtcgcgcaagaagtccgtgactgtctcggttccgaataccggttcggtgacatccagagtgggtgtggtcttggggatcgctggtgaagtcatgagagtacctttcgtgt
gagtacgggtgatggtgctgttcctctatttccgcaagccgaattccagcacactggcggccgttactagtggatccgagctcggtaccaagcttggcgtaatcatggtcat
agctgtttcctgtgtgaaattgttatccg
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BIOGRAPHICAL SKETCH

Mr. Anhui Huang graduated from No. 7 High School in Quanzhou, P.R.China, in 2002, and then entered the College of Life Sciences at Nankai University for his undergraduate study. In 2003, He was admitted to the National Training Base in Life Science and Technology in Nankai University, where he had my specific education and training in DNA recombination technology, microbial engineering and biochemical engineering. He received his bachelorous degree in Biotechnology in 2006 and has been a graduate student in Soil and Water Science Department at the University of Florida since then. He is scheduled to obtain his Master of Science degree in May 2009.