

INTRODUCING THE
ArsR REGULATED ARSENIC STIMULON

by

Tara Carlyne Saley

A thesis submitted in partial fulfillment
of the requirements for the degree

of

Master of Science

in

Land Resources and Environmental Science

MONTANA STATE UNIVERSITY
Bozeman, Montana

July 2017

©COPYRIGHT

by

Tara Carlyne Saley

2017

All Rights Reserved

ACKNOWLEDGEMENTS

I would like to thank my advisor Dr. Timothy McDermott for sharing his extensive knowledge of microbial-arsenic interactions, his guidance in performing the research, and his help in preparing and reviewing this thesis. Credit is also due to my committee made up of Dr. Brian Bothner, Dr. Ross Carlson, and Dr. Anthony Hartshorn for their input in the research and thesis writing processes. Thank you to National Science Foundation for funding the research.

TABLE OF CONTENTS

1. LITERATURE REVIEW	1
Introduction to Arsenic	1
Environmental Chemistry of As	1
Abiotic Environmental Conditions	2
Microbial Effects	3
Sources of As	5
Minerals and Rocks.....	5
Soils.....	5
Water.....	7
Atmosphere	10
Geothermal Areas	11
Microbial Bioremediation of As	12
Biostimulation.....	13
Bioaugmentation	13
Risks of As Exposure.....	13
Risks to Humans	14
Microbe-As Interactions.....	15
<i>ars</i> Operon	16
Regulatory Proteins.....	16
As (III) Transporters	18
As (V) Reductases.....	19
Additional Proteins	20
<i>aio</i> Operon	21
Signal Transduction	22
AioAB.....	22
Additional Known Requirements	23
<i>pho</i> <i>Regulon</i>	23
Signaling System	23
<u><i>pstSCAB-phoU</i> operon.</u>	24
As Metabolism Genes	24
2. INTRODUCING THE ArsR-REGULATED ARSENIC STIMULON	26
Introduction.....	26
Materials and Methods.....	29
Reporter Constructs and Deletion Mutants.....	28
Bacterial Growth Conditions	30
<i>arsR</i> Reporter Assays.....	31
RNA Extraction	31
RNA Sequencing	32

TABLE OF CONTENTS CONTINUED

Sequence Analysis	32
Results	33
<i>arsR</i> Reporter Assays.....	33
Gene Expression Changes Related to As(III) Exposure	35
Evaluating Changes in Gene Expression Levels of the <i>arsR</i> Mutants.....	41
Genes Potentially Co-Regulated by All Four ArsR Proteins.....	46
Genes Regulated by ArsR1 Only	47
Genes Regulated by ArsR2 Only	49
Genes Regulated by ArsR3 Only	54
Genes Regulated by ArsR4 Only	55
As(III) Influences on ArsR Regulatory Control	57
Arsenic Resistance	58
Chemotaxis	58
Sugar Transporters	59
Iron Homeostasis	60
Discussion	61
Purpose of <i>arsR</i> Reiteration	62
Activator Function	64
Functions Regulated by the ArsR Proteins	65
Significance.....	70
REFERENCES CITED.....	71

LIST OF TABLES

Table	Page
1. Bacterial strains and constructs used in this study.....	30
2. Significant gene expression changes in wild type <i>A. tumefaciens</i> as a function of As(III) exposure	37
3. Summary of functions influenced by ArsR proteins.....	45
4. Genes exhibiting expression patterns suggesting they are influenced directly or indirectly by all of the ArsR proteins	47
5. Gene expression significantly influenced by ArsR1 only	48
6. Gene expression significantly influenced by ArsR2 only	51
7. Gene expression significantly influenced by ArsR3 only	54
8. Gene expression significantly influenced by ArsR4 only	56
9. Influence of As(III) on ArsR control of the arsenic resistance response	58
10. Influence of As(III) on ArsR control of chemotaxis.....	59
11. Influence of As(III) on ArsR control of sugar transporters	60
12. Influence of As(III) on ArsR control of iron homeostasis.....	61

LIST OF FIGURES

Figure	Page
1. As speciation based on environmental conditions	3
2. As in the top 0-5 cm of soil in the United States	6
3. As levels in drinking water around the United States	9
4. As Levels in Gallatin County, MT.....	10
5. Representations of the <i>arsR1</i> and <i>arsR2</i> loci in <i>A. tumefaciens</i>	17
6. Organization of the <i>arsR1</i> and <i>arsR2</i> loci.....	34
7. Reporter gene assays monitoring expression of <i>arsR1</i> , <i>arsR2</i> , and <i>arsR4</i> as a function of As(III) exposure and time.....	35
8. Volcano plots illustrating the influence of each ArsR on genome-wide transcription in the absence of As(III)	43
9. Genes uniquely repressed by ArsR1	49
10. ArsR regulatory hierarchy.....	63
11. Functions regulated by the ArsR proteins with no As(III)	66

ABSTRACT

The United States EPA ranks arsenic as the number one environmental toxin. Since microorganisms are significant drivers of arsenic toxicity and mobility in nature, it is important to understand how microbes detect and react to arsenic. The microbial arsenic resistance operon (*ars*) is critical for sensing arsenic in the environment and controlling the cellular response to this toxin. The *ars* operon is minimally comprised of *arsRBC*, which codes for an ArsR transcriptional repressor, arsenite effluxer, and an arsenate reductase, respectively, with the operon negatively regulated by the transcriptional repressor, ArsR. Our model organism *Agrobacterium tumefaciens* 5A carries two *ars* operons, with each containing two *arsR* genes. We conducted an RNASeq study to examine the regulatory roles of the encoded four ArsR regulatory proteins as a function of +/- arsenite. We report that the regulatory influence of the ArsR proteins extends well beyond the *ars* operon, with both activation and repression effects. In addition to the expected arsenic resistance response, many cellular functions were impacted, including: phosphate acquisition/metabolism, sugar transport, chemotaxis, copper tolerance, and iron homeostasis. Each of the ArsR proteins uniquely influenced different sets of genes and an *arsR* regulatory hierarchy was observed, wherein ArsR1 is auto regulatory and negatively regulates *arsR4*, ArsR4 activates *arsR2*, and ArsR2 negatively regulates *arsR3*. ArsR3 is the least active with respect to number of genes regulated. To summarize, this study provides a more complete understanding of how microbial gene expression and biogeochemical cycling may be influenced by arsenic in the environment.

LITERATURE REVIEW

Introduction to Arsenic

Arsenic (As) is at the top of the toxic substance priority list prepared by the ATSDR and the EPA because of the frequency, toxicity, and potential for human exposure at national priority list sites (ATSDR, 2016). The World Health Organization has ranked As among the top ten chemicals that threaten human health (WHO, 2016). While often grouped with heavy metals, As is actually a metalloid meaning it has both metallic and nonmetallic properties. Organic As compounds are molecules wherein As is directly bonded with carbon, whereas inorganic As is bonded with elements such as oxygen or sulfur and are usually more harmful to various forms of life (Kaur, Kamli, & Ali, 2011). As hotspots exist around the world. Some areas may have naturally high levels of As, but anthropogenic sources can contribute substantially to As contamination in soils and waters. Once As has entered an environment, it cannot be destroyed but may change forms or solubility through the actions of bacteria or prevailing environmental chemistry. This literature review will cover sources of As, environmental chemistry of As, risks of As exposure, and microbe-As interactions.

Environmental Chemistry of As

As can be found in oxidation states of +5, +3, 0, and -3 (Kaur et al., 2011). It is typically found in the environment as arsenite [As(III)] and arsenate [As(V)], whereas elemental As [As(0)] and arsine [As(-III)] are not as common. Speciation of As determines its mobility and toxicity. As(V) tends to be less mobile and less toxic,

whereas As(III) is more mobile and more toxic. Speciation is determined by redox potential, pH, and microorganism activity (Kang, Heinemann, Bothner, Rensing, & McDermott, 2012; Kruger, Bertin, Heipieper, & Arsene-Ploetze, 2013).

Abiotic Environmental Conditions. Both redox potential and pH of the environment are abiotic factors that influence As speciation (Figure 1). A redox reaction occurs when an electron is passed from one atom to another. The atom that accepts the electron is reduced and the atom that donates the electron is oxidized. Some environmental conditions are conducive to reducing and some are conducive to oxidizing. Redox conditions play a major role in As speciation, with As(V) usually being the dominant species in aerobic conditions and As(III) being dominant under anaerobic conditions. For example, in oxidizing aquatic environments, As(III) is rare, while the majority of As exists as As(V) (Kruger et al., 2013). Redox conditions can also change As solubility by affecting binding sites in soil, which are often oxidized forms of iron, manganese, or aluminum. Both As (V) and As(III) can form complexes with metal oxides, but As(V) is more strongly sorbed at near-neutral pH (such as in most groundwater). As(V) is often associated with iron-(oxy)hydroxides. In oxidizing conditions, the hydroxide is stable and remains in solid state, along with the sorbed As(V). In reducing conditions, the hydroxide becomes less thermodynamically stable and may release to the solution along with the As (Smedley & Kinniburgh, 2002). A major example of this phenomenon is in the Bengal Basin. Reducing conditions were likely the reason for the As contamination in groundwater of this region. It is likely that this As

contamination was in part due to reductive desorption of As from oxides and clays (Smedley & Kinniburgh, 2002).

pH also plays a major role in As speciation in the environment, causing As to lose or gain H^+ . When pH increases, As groups will lose H^+ and as pH decreases As groups will gain H^+ , and will affect the solubility of As. When pH increases, As(V) becomes more negatively charged, which in theory should allow for stronger bonds to the positive soil binding sites. However, soil binding sites are also affected by pH, with OH^- groups from water increasing as pH increasing.

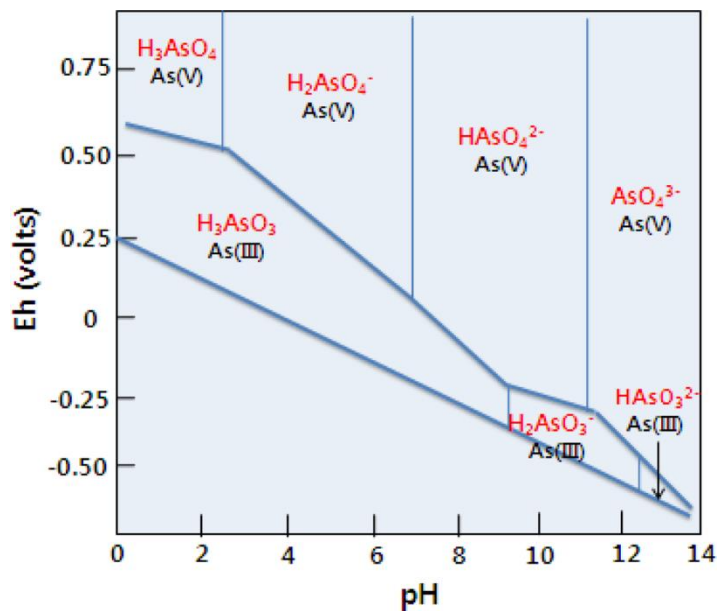


Figure 1. As Speciation Based on Environmental Conditions. Certain Species of As are dominant at different redox potentials and pH (Lim et al. 2009).

Microbial Effects. Microbes survive and thrive in many high As areas because they have the potential to resist and metabolize As. Through these activities microbes can change the speciation of As. Microbial speciation of As may occur for many reasons

including: dissimilation, assimilation, regulation of reducing/oxidizing equivalents, and detoxification (J. F. Stolz & Oremland, 1999). As(V) reduction and As(III) oxidation are both performed by microbes, and contribute significantly to biogeochemical As cycling.

As(V) can be reduced for energy generation or detoxification purposes. The *ars* operon contains genes that work to detoxify As in bacterial cells. The As(V) reduction occurs after As(V) has entered the cell through a phosphate transporter. Once in the cell, As(V) is reduced to As(III) through the ArsC protein. Then As(III) is extruded from the cell with the help of ArsAB. As(V) can be reduced to As(III) for energy generation by acting as a terminal electron acceptor. This is termed arsenate respiration and has been reported in several different prokaryotes (J. E. Stolz, Basu, Santini, & Oremland, 2006).

As(III) may also be oxidized for detoxification or energy generation purposes. As(III) oxidation for detoxification occurs because As(V) is less toxic than As(III). As(III) oxidation can also be used for energy generation where bacteria use As(III) as a sole energy source (Santini, Sly, Schnagl, & Macy, 2000). As(III) oxidation leads to energy generation by coupling the oxidation of As(III) to reduction of oxygen or nitrate (e.g., Rhine et al., 2006; Zagar et al. 2010; Zhang et al. 2015). The biotic As oxidation rate is up to two orders of magnitude higher than the abiotic oxidation rate (Gihring & Banfield, 2001). The genetics and physiology of microbe-arsenic interactions will be discussed in greater detail below.

Sources of As

Minerals and Rocks. As is naturally distributed in the Earth's crust and ranks 20th in elemental abundance (Mukherjee & Bhattacharya, 2001), but is not distributed uniformly. Igneous rocks typically contain low levels of As, sedimentary rocks usually have a higher As concentration than igneous rocks, and metamorphic rocks generally have around the same As concentration as the igneous or sedimentary rocks that were their precursors (Smedley & Kinniburgh, 2002). These rocks can act as parent material and thus can influence As levels in soils (see below).

Considering rock composition, there are over 200 As-containing minerals. Most As-containing minerals are often found in conjunction with transition metals such as gold, antimony, phosphorus, molybdenum, cadmium, lead, and silver. As can be found in many rock-forming minerals, but the most common As-associated mineral is arsenopyrite (FeAsS), wherein As has replaced sulfur in the crystal structure. As can also be found in high quantities in hydrous metal oxides and oxide minerals, either as part of the structure or sorbed species. In phosphorus (P)-containing minerals, As may be present in high quantities (Smedley & Kinniburgh, 2002).

Soils. Common As levels in soils are often around 5-10 ppm, varying due to soil parent material and anthropogenic sources (Smedley & Kinniburgh, 2002). Throughout the United States there are soils that have high As contamination due to both (Figure 2), posing a risk to human health through contact/ingestion of the soil directly or through contamination of drinking water sources.

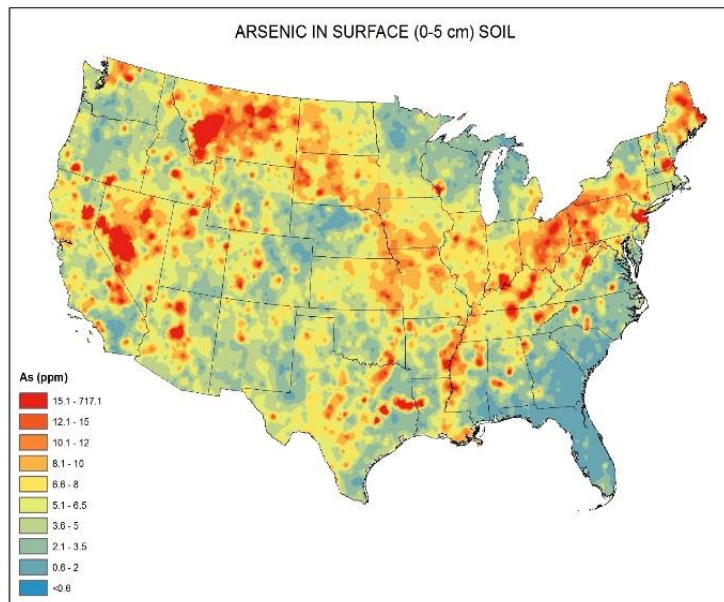


Figure 2. As in the top 0-5 cm of soil in the United States. Red indicates the highest levels of As contamination (USGS, 2005).

As containing insecticides are a major source of contamination in many agricultural areas. These pesticides have been around for centuries, with evidence of As sulfides being used in China around 900A.D. Copper acetoarsenite, also known as Paris green, was first used in 1867 in the United States and used consistently until the 1900's by fruit growers to control moths. Lead arsenate was the most widely used arsenical insecticide and was utilized all over the US, Australia, Canada, New Zealand, France, North Africa, and England (Peryea, 1998). It was banned in the US in 1988 (Schooley, 2008), though the legacy of its use remains because once introduced As remains as compared to carbon-based contaminants which microbes can mineralize to CO_2 . In agricultural soils, As concentrations have been reported to reach 366-732 mg kg^{-1} (Ure &

Berrow, 1982). It is estimated that today over 188,000 acres in Washington have As contaminated soil due to lead arsenate pesticide use in apple orchards (Hood, 2012).

Another major use of As was for Chromated Copper Arsenate (CCA). CCA was widely used as a wood preservative in residential applications. Millions of cubic meters of CCA-treated wood were produced annually in the United States until a voluntary ban of its use began in 2003 for residential areas due to health concerns. Its use in residential areas include but are not limited to: docks, telephone poles, decks, picnic tables, and playground equipment (Khan, Solo-Gabriele, Dubey, Townsend, & Cai, 2004). Currently, new CCA application in residential areas is banned, but As is used in other areas such as marine and industrial applications. CCA has been shown to leach As into soils during weathering (Gress et al., 2016).

Mining-impacted soils and sediments can contain extremely high As levels, sometimes being reported to contain up to several thousand mg kg^{-1} (Smedley & Kinniburgh, 2002). These concentrations are a result of increased iron arsenates and iron oxides that are a product of ore minerals as well as an increase in the exposure of As-rich sulphide minerals to weathering processes (Smedley & Kinniburgh, 2002). As is also a byproduct from smelting process of many metals including: cobalt, gold, lead, nickel, and zinc (ATSDR, 2011). Metal smelting produces slag and sludge that contain As that are usually dumped in a landfill (Chilvers, 1987).

Water. As concentrations in water range from $0.5 \mu\text{g l}^{-1}$ to more than $5000 \mu\text{g l}^{-1}$, although in general less than $10 \mu\text{g l}^{-1}$ (Smedley & Kinniburgh, 2002). Certain environmental conditions lead to higher As water concentrations including strongly

reducing conditions at neutral pH that leads to desorption of As from mineral oxides (Smedley & Kinniburgh, 2002). Waste water from various sources can lead to As contamination; e.g., metal smelting can lead to wastewater with high concentrations of As (Chilvers, 1987). Mining process may lead to leaching of As from metal ores in acid rock drainage (Williams, 2001).

Most groundwater contamination is due to geology of the area. There are many areas that have naturally high As concentrations in groundwater including Argentina, Bangladesh, Chile, China, India, Mexico, and the US (WHO, 2012). The As contamination issues of groundwater in Bangladesh caused what is referred to as the largest poisoning of a population in history. There were an estimated 35 to 77 million people at risk. In this case, drinking water was primarily obtained through tube-wells that drew from aquifers contaminated with As. A large percentage of tube-wells were found to have As concentrations above 50 µg/l, which is considered a public health emergency (Smith, Lingas, & Rahman, 2000). The United States also has contamination issues with As in drinking water (Figure 3).

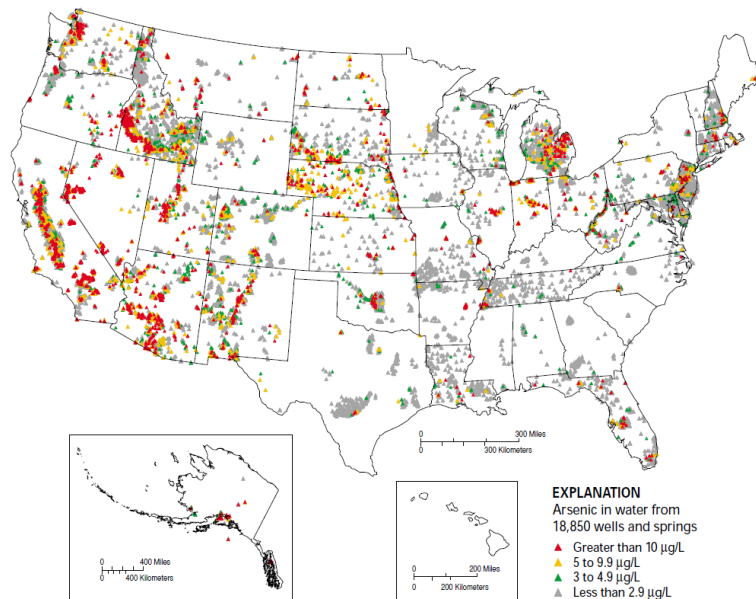


Figure 3. As levels in drinking water around the United States. Red indicates As levels over the EPA limit (Focazio, 2000).

Closer to home, well testing in Gallatin county in Montana showed that several wells were above (sometimes significantly above) the drinking water standards (Figure 4). These high As levels are due to the natural geology of the area. Often drinking water from private wells are not tested for As levels and residents may unknowingly be drinking water with As well above the EPA's standard of 10 µg/L. High As in drinking water is a relevant concern throughout the US, due to both anthropogenic and natural geological As sources.

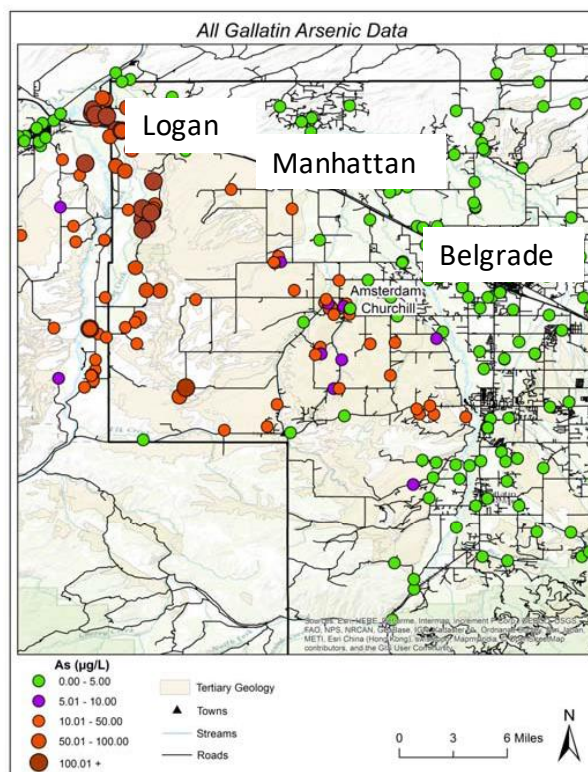


Figure 4. As levels in Gallatin County, MT/ As concentration was tested in private wells. Any red or brown dot is above the EPA drinking water standard (Miller, 2017)

Atmosphere. Natural concentrations of As in the atmosphere are usually low but volcanic activity, wind erosion, volatilization from soils, marine aerosols, and anthropogenic pollution may contribute to contamination. Most of the As in the atmosphere is particulate matter (Smedley & Kinniburgh, 2002). Volcanic activity is the greatest natural source of As emissions to the atmosphere, contributing an estimated 25% of As atmosphere emissions. Other natural sources and in order of relative contribution, include wind erosion, forest fires, low temperature volatilization, and sea spray (Chilvers, 1987).

It is estimated that anthropogenic sources of As make up 70% of the global atmospheric As flux (Nriagu & Pacyna, 1988). Smelter operations and fossil-fuel

combustion are the most significant anthropogenic inputs of As into the atmosphere. Concentrations of As in coal are often very high, sometimes as high as 35,000 mg kg⁻¹ (Finkelman, Belkin, & Zheng, 1999). In coal-smoke polluted areas, As levels in the atmosphere are higher than in non-polluted areas. As air concentrations in cities have been measured around 0.003 - 0.18 µg m⁻³, with levels close to industrial emissions being reported exceeding 1 µg m⁻³ (WHO, 2001). There is concern about inhalation of As through coal-smoke may be linked to lung cancer, especially in highly industrialized areas such as the Guizhou Province in China (Finkelman et al., 1999).

Geothermal Areas. Geothermal areas can have extremely high levels of As. Geothermal areas can occur due to rift zones (where tectonic plates are separating), “hot spots” (where magma chambers are near the Earth’s surface), and tectonic plate boundaries. Geothermal waters contain dissolved As that mainly derives from host rock leaching. Levels reported in different studies include (but are not limited to) : Yellowstone National Park with concentrations up to 14.6 mg/L (McCleskey, Nordstrom, Susong, Ball, & Taylor, 2010), El Tatio Geyser Field with concentrations of up to 33.7 mg/L (Landrum et al., 2009), and Sebgan geothermal areas in Japan with concentrations up to 13 mg/L (Pascua, Minato, Yokoyama, & Sato, 2007). Surface waters draining geothermal systems can have high As concentrations. For example, As levels have been measured up to 270 µg/L in the Madison River near West Yellowstone, MT (Nimick, Moore, Dalby, & Savka, 1998). There are few examples of geothermal areas contaminating groundwater because vertical ascension of the water limits opportunities

for lateral-spreading of these waters at depth. However, near surface or shallow aquifer As contamination can occur (Webster & Nordstrom, 2003).

Microbial Bioremediation of As

Depending on what is suitable for the As bioremediation method, microbes can be used to both mobilize and immobilize As. For immobilization, microbes can directly oxidize the As(III). Alternatively, they can oxidize Fe(II) to form Fe(III) that then becomes a strong sorbent for As(V). This general principle can be used and enhanced in several ways to efficiently remediate As. If the goal is to mobilize As to remove it from the soil, microbes can reduce the As(V) or they can reduce Fe(III).

Determining which microbes occur at remediation sites is an important step in microbial bioremediation. Microbe populations can be characterized using molecular biological tools (MBTs), quantitative polymerase chain reactions (qPCR), microassays, and/or fluorescence *in situ* hybridization (FISH). These techniques can help determine if the site is a candidate for bioremediation with microbes. Intrinsic bioremediation involves the use of naturally occurring microbes, allowing them to act upon As naturally without any aid/interference from humans. Engineered bioremediation is when humans create an environment that is more ideal for the extant microbe or by adding microbes to the environment (Lim, Shukor, & Wasoh, 2014). Engineered bioremediation is divided into biostimulation or bioaugmentation, and are employed at sites where native bacteria may not be able to successfully remediate contaminants (EPA, 2013).

Biostimulation. In biostimulation an electron donor or acceptor is added to a remediation site in order to stimulate the actions of resident microbes (EPA, 2013). Anaerobic zones are conducive to the reduction of both Fe and As, leading to their release into the water. By contrast, enhancing aerobic conditions (e.g., addition of H_2O_2) will lead to the oxidation of both Fe(II) and As(III), thereby resulting in stable sorption of the As(V) and reducing its toxicity and mobility.

Bioaugmentation. Bioaugmentation is when microbes are inoculated into a system to break down a contaminate (EPA, 2013), allowing the addition of microbes tailored to the remediation goal. The microbes chosen must be fit to survive in the environmental conditions with considerations such as temperature, pH, predators, competitors, nutrients, and more (Sprocati et al., 2012). The goal is maximizing survival of the microbes that will provide bioremediation services.

Risks of As Exposure

As(V) is a phosphate (P) analog meaning it has a similar structure. Because of the structural similarities, As(V) can enter the cell through P transporters and can cause many problems in a cell. P has many essential roles including being part of DNA, RNA, and ATP (the “energy currency” of the cell). P plays many roles in processes crucial to life. If As(V) enters the cell, it can interfere with these processes (Rosen, Ajees, & McDermott, 2011).

As(III) is around ten times more toxic than As(V), and will enter cells through aquaglyceroporins. It attacks thiols and thus inhibits enzymes that rely on thiols (-SH). If

As(III) binds to an important thiol group, it may prevent crucial biochemical events in the cell from occurring which can cause toxicity (Hughes, 2002). As(III) may also degrade Fe-S clusters in proteins (Kruger et al., 2013).

Risks to Humans. Upon exposure, most As is excreted through nails, breath, stool, urine, and skin. If the intake is excessive, it can cause multiple negative effects in the human body. Inorganic As is a “class A” carcinogen and is a potential endocrine disruptor (Stilwell, 2015). As can react with sulfhydryl groups of proteins and enzyme and substitute for P in many different biochemical reactions, decreasing the efficacy of enzymes. As can cause impairment of cellular respiration through inhibition of mitochondrial enzymes and uncoupling of oxidative phosphorylation. As can also inhibit DNA repair, induce chromosomal aberrations, and cause sister-chromatid exchanges. As(III) compounds induce gene amplification, arrest cells in mitosis, and act as promoters and co-mutagens with other toxic substances (Tchounwou, 2003).

Dermal effects of As exposure include spotted hyper and hypo- pigmentation, palmar hyper-keratosis, and plantar hyperkeratosis. Cardiovascular effects of As include hypertension, an increase in cardiovascular disease mortality, increase in coronary heart disease, blackfoot disease, cardiovascular disease, bronchitis, polyneuropathy, peripheral vascular disease, and noncirrhotic portal fibrosis. Reproductive and developmental effects of As exposure include potential increase in abortions, congenital malformations, and increase in stillbirths. Respiratory effects include bronchopulmonary disease and chronic obstructive pulmonary disease. Chronic exposure to As can affect heart, nervous system, liver, skin, gastrointestinal tract, and skin. Carcinogenic effects of As include

increased risk of lung cancer for inhaled As, skin cancer and internal cancers including liver, kidney, lung, colon and bladder when exposed orally (Tchounwou, 2003). Large oral doses of As (over 60 ppm) can result in death. Lower levels, around 300-30,000 ppb in water may cause symptoms such as stomach ache, nausea, vomiting, and diarrhea. Oral dose tests have shown that skin lesions begin around exposure levels of 0.002-0.02 mg As/kg/day, and some studies indicate that even lower levels may cause dermatitis (*Toxicological Profile for Arsenic*, 2007).

Microbe-Arsenic Interactions

Microbes have several ways of dealing with As in their environment. Some are able to utilize As in metabolic processes, some can detoxify As, and some are capable of doing both. Many microbial metal-survival techniques involve change of speciation that leads to changes in mobility and toxicity. Thus, microbial As-related processes have a major role in As biogeochemical cycling (Gadd, 2010). Microbe-As interactions are coordinated by various genes that are organized into operons. Functions of As related operons include As(III) extrusions from the cell (*ars* operon), As(III) oxidation (*ai* operon), respiratory As(V) reduction (*arr* operon), and photosynthetic As(III) oxidation (*arx* operon) (Branco, Chung, & Morais, 2008). Genomic techniques studying As-microbe interactions improve our understanding of processes at work in nature and allow for more effective remediation strategies.

ars Operon

The arsenic resistance (*ars*) operon is the most thoroughly studied microbe-As interaction operon (van Lis, Nitschke, Duval, & Schoepp-Cothenet, 2013). *ars* genes provide the organism with protection from As

(III) and As(V). The *ars* genes are organized into operons that are commonly contain either three or five genes (*arsRBC* or *arsRDABC* respectively) (Butcher & Rawlings, 2002). *arsRBC* is the simplest *ars* operon that allows for As resistance. It includes genes for a regulatory protein (ArsR), a As(V) reductase (ArsC), and an extrusion protein (ArsB). In the five gene operon, ArsD is a metallochaperone and ArsA can form a complex with ArsB to utilize ATP and increase As extrusion efficiency.

The *ars* operon is activated after As enters the cell. As(III) can enter through aquaglyceroporins. As(V) can enter the cell through a phosphate (P) transporter as it is a molecular analog of P (Oremland & Stolz, 2003). When As(V) enters the cell, it is enzymatically reduced to As(III) by the enzyme As(V) reductase. The As(III) in the cell is then actively removed from the cell through an efflux pump. Many combinations for these genes have been found and novel genes, orthologues, and gene functions in the *ars* operon are still being identified in various organisms.

Regulatory Protein. ArsR is a responsive regulatory protein, and traditionally viewed as being a typical representative of the ArsR family of proteins; i.e., a DNA binding repressor. However, recently activator activity has been indicated for the ArsR proteins (Kang et al., 2016). When As(III) enters a cell and binds the ArsR protein, the protein changes conformation and releases from the DNA, allowing transcription to

occur. In the bacterium used for this study, *A. tumefaciens*, there are two different *ars* loci that contain four *arsR* genes named *arsR1*, *arsR2*, *arsR3*, and *arsR4* (Figure 5) and that encode their respective proteins (e.g., ArsR1). ArsR1 and ArsR4 are more closely related displaying 93% identity and 96% similarity, but differ substantially from ArsR2 and ArsR3 which share 78% identity and 86% similarity. Kang et al. (2016) used *lacZ* reporter constructs to examine the expression of *arsR1*, *arsR2* and *arsR4*, but needed to use reverse transcriptase PCR to assess *arsR3* expression because this gene is part of an operon and transcriptionally linked to an upstream gene, *lysR*. The *lacZ* constructs were used with the different *arsR* deletion mutations to evaluate regulation of the *arsR* genes. The study found *arsR1*, *arsR2*, and *arsR4* are inducible by As(III). However, expression of *arsR3* did not appear to be linked to As(III). ArsR1 and ArsR2 were found to be autoregulatory (repress their respective encoding gene), whereas Ars4 is not but rather is influenced by ArsR1 (Kang et al., 2016).

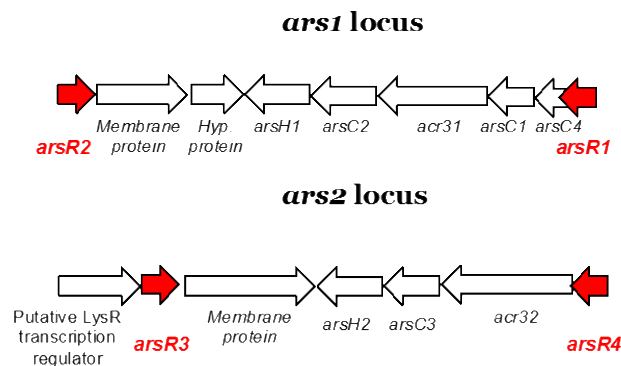


Figure 5. Representation of the *arsR1* and *arsR2* loci in *A. tumefaciens*. The *arsR* genes are in red (Modified from Kang, 2016).

As(III) Transporters. ArsD is a protein that was initially thought to play a small role in the cell as trans-acting regulator protein until its function as a metallochaperone was discovered (Butcher & Rawlings, 2002). The regulation role of ArsD is to control upper-level expression of the *ars* operon. ArsD and ArsR bind to the same operator site but not at the same time depending on expression. However, ArsD has a lower affinity for the binding site (up to two orders of magnitude less) than ArsR so it is not a high efficiency regulator (Kaur et al., 2011). ArsD increases the affinity of ArsA for As(III) by acting as a metallochaperone and shuttling the As(III) to the ArsA subunit of the ArsAB efflux pump. ArsD and ArsA are always found together in operons or gene clusters (Lin, Walmsley, & Rosen, 2006).

There are two main methods of extruding As(III) from the bacterial cell. One method uses membrane potential of the cell to efflux through an As(III) carrier protein (ArsB and Acr3) and the other uses energy (ArsAB) (Pillai, Venkadesh, Ajees, Rosen, & Bhattacharjee, 2014). The ArsB protein is hydrophobic and has 12 membrane-spanning α -helices (Dey, Dou, Tisa, & Rosen, 1994). ArsB can act independently as a passive translocator or as membrane anchor and substrate-conducting pathway (Pillai et al., 2014). ArsA is a hydrophilic membrane protein that is a catalytic subunit for the efflux pump (Kaur et al., 2011). There are two subunits to ArsA, A1 and A2, which are linked by a 25-residue linker and are both necessary for activity. ArsA is activated allosterically to hydrolyze ATP by the presence of As(III) or Sb(III). The hydrolysis of ATP by ArsA is coupled to movement of As(III) or Sb(III) to ArsB (Dey et al., 1994). It is usually found bound to ArsB but can also be in the cytosol if ArsB is not present. The

ArsAB system is more efficient than ArsB by itself and thus cells with the ArsAB system are more resistant to As (Rosen, 2002).

The Acr3 protein is a transporter that has a similar function to ArsB but little amino acid sequence similarity. There are two families of Acr3 based on phylogenetic differences: Acr3-1p and Acr3-2. Acr3-1 is the more active As(III)/H⁺ antiporter (Kang, Shi, Bothner, Wang, & McDermott, 2015). An organism may have only one or may have both *acr3* and *arsB* genes. In a 2009 study, it was found that Acr3-1 and Acr3-2 were associated with high and moderate levels of As resistance, whereas ArsB was associated with high, moderate, and low levels of As (Cai, Liu, Rensing, & Wang, 2009). It was also suggested that Acr3 may be more efficient at extruding As than ArsB (Cai et al., 2009).

As(V) Reductases. Earth is about 4.5 billion years old, with estimates of life beginning on Earth approximately 3.5-3.8 billion years ago. There was likely more As on earth's surface at the beginning of the Archean (about 3.8 Ga) than there is today, which provided opportunities and challenge for emerging life (Oremland, Saltikov, Wolfe-Simon, & Stolz, 2009). The environment on early earth was very different from the environment today, with a major difference being that there was no oxygen in the atmosphere. Thus, the organisms survived through anaerobic metabolism (Schlesinger, 2013). In these early conditions, As(III) would have been the dominant species of As in solution and thus earliest microbial As resistance methods would likely have targeted As(III) through an extrusion mechanism. At approximately 2.45 to 2.32 billion years ago, Earth's atmosphere became oxic, termed the Great Oxidation Event, with the new redox conditions favoring the formation of As(V). Consequently, microorganisms needed to

develop detoxification strategies for As(V). Based on contemporary microbial genetics, it appears an As(V) reductase enzyme evolved to convert the As(V) to the substrate for the extrusion pump. Thus, proteins evolved to reduce As(V) to As(III) (Rosen, 1999). This As(III) could then be extruded using the already developed As(III) extrusion mechanisms (Rosen, 2002).

There are two different families of the ArsC protein. The first family of ArsC reduces As(V) using glutaredoxin and glutathione as electron sources (Martin et al., 2001). This family has a single catalytic cysteine (Lopez-Maury, Sanchez-Riego, Reyes, & Florencio, 2009). The next family of ArsC is unrelated to the other family. The ArsCs in this family are protein phosphotyrosine phosphatases. This family includes *Staphylococcus aureus* and *Bacillus subtilis* As(V) reductases. Thioredoxin is the source of reducing potential and three cysteine residues are utilized (Messens & Silver, 2006). Acr2p is a less common reductase that uses glutaredoxin and glutathione as reluctant (Rosen, 2002).

Additional Proteins. ArsM and ArsI control As methylation and demethylation. ArsM is an As(III) S-adenosylmethionine methyltransferase. It is widespread with representative in members of every kingdom (Zhu, Yoshinaga, Zhao, & Rosen, 2014). ArsM can methylate As(III) to the volatile trimethylarsine, which escapes the cell and thus eliminating toxicity (Qin et al., 2006). ArsI is responsible for demethylation of organoarsenicals. ArsI is a nonheme iron-dependent dioxygenase with C⁺ As lyase function that is responsible for transforming methylated As(III) back to its inorganic form

[As(III)]. It is important as it allows organisms to have resistance to organoarsenicals as well as inorganic As (Yoshinaga & Rosen, 2014).

Recent research has shown that ArsH is an organoarsenical oxidase that oxidizes trivalent methylated and aromatic arsenicals to pentavalent species. This confers resistance to environmentally methylated and aromatic arsenicals (Chen, Bhattacharjee, & Rosen, 2015). *arsH* is widespread in bacteria, and some fungi (Ye, Yang, Rosen, & Bhattacharjee, 2007).

Additional *ars* genes that perform functions that are thought to be involved in As resistance include: *arsTX*, *arsO*, *arsP*, and *arsN*. ArsTX is a thioredoxin system transcribed along with an *arsRC2* fusion gene (Achour-Rokbani, Cordi, Poupin, Bauda, & Billard, 2010). ArsO is a putative flavin-binding monooxygenase (Wang et al., 2006). ArsP contributes to resistance of organic As compounds by acting as a efflux transporter as studied in *C. jejuni* (Shen et al., 2014). ArsN is similar to acetyltransferases that is closely associated with arsenic resistance genes and has been shown to contribute resistance to sodium As(V) (Chauhan, Ranjan, Purohit, Kalia, & Sharma, 2009).

aio Operon

The *aio* operon is involved in As(III) oxidation. As(III) oxidation may occur for energy generation or detoxification purposes. As(III) oxidation for detoxification occurs because As(V) is less toxic than As. As(III) oxidation leads to energy generation by coupling the oxidation of As(III) with the reduction of oxygen or nitrate (Zargar, Hoeft, Oremland, & Saltikov, 2010). In most bacteria, a signal transduction system made up of AioX, AioS, and AioR regulate the expression of AioAB (Shi, 2017), the genes that

encode the As(III) oxidase enzyme. Through the use of genomic screening, it has been shown that *aio* operon is widespread in bacteria and archaea (van Lis et al., 2013).

Signal Transduction. AioS and AioR are a two-component signal transduction pair that are involved in regulating microbial As(III) oxidation (Liu et al., 2012). AioS acts as the sensor kinase and AioR acts as the cognate regulator. AioS transfers a signal to its core through a periplasmic sensory domain attached to transmembrane helices to HAMP domain. AioR is a transcriptional regulator that interacts with DNA through a helix-turn-helix domain (Sardiwal, Santini, Osborne, & Djordjevic, 2010). AioX is a periplasmic As(III)-binding protein. It has been shown to play an essential role in *aioAB* expression, likely sensing or transducing the As(III) signal. This may occur through As(III) being bound and then transferred to AioS, or AioX undergoing a conformational change that allows it to interact with AioS to initiate a signaling pathway (Liu et al., 2012).

AioAB. AioAB is a heterodimeric enzyme that oxidizes As(III) to As(V) (Cordi et al., 2015). AioAB is a Mo-containing DMSO reductase (McEwan, Ridge, McDevitt, & Hugenholtz, 2002). AioA is the large subunit and is the catalytic element that contains a molybdenum-bis co-factor and has a [Fe-S] core and consists of four domains (Koechler et al., 2010). The catalytic pocket has highly conserved amino acids (IHNRPAYNSE) with a non-coordinating alanine (J. E. Stolz et al., 2006). The AioA and AioB subunits are held together by AioA's C- and N- stretches that wrap around AioB protein and hydrogen bonds between the interface of the two subunits (Ellis, Conrads, Hille, & Kuhn, 2001).

Additional Known Requirements. RpoN is a sigma factor that plays a role in initiation of AioAB transcription. RpoN is specific for a -12/-124 binding site (Koechler et al., 2010). DnaJ is protein that has been shown to assist in protein folding and mRNA stability and is required for *aioAB* transcription. The exact role of DnaJ in *aioAB* transcription is not known but some suggestions for its role include folding of AioR or chaperoning proteins (Koechler et al., 2010). A molybdate transporter and a Na^+/H^+ antiporter are also essential for As(III) oxidation (Kashyap, Botero, Lehr, Hassett, & McDermott, 2006).

pho Regulon

The Pho regulon is made up of a large number of genes that are involved in the cells response to P starvation. Inorganic orthophosphate, P_i , is the preferred P source for bacteria. The *aio* signaling system is sensitive to inorganic P. PhoB1 and PhoB2 proteins are partially responsible for regulation of the Aio system (Kang, Bothner, Rensing, & McDermott, 2012). Thus, the Pho regulon plays an important role in cell As(III) oxidation. When the *pho/pst/phn* genes are located adjacent to *aio* system, the *aio* genes can be expressed in response to P starvation (Kang, Heinemann, et al., 2012). There are seven genes that are required for P_i signaling which will be covered below (Hsieh & Wanner, 2010).

Signaling System. PhoB and PhoR act a two component signaling system. PhoR is a membrane signaling histidine kinase. PhoR interacts with a P transport system in response to orthophosphate concentrations (Lamarche, Wanner, Crepin, & Harel, 2008).

PhoR activates PhoB during P_i limitation, acting as a two-component signaling system. PhoR may prevent phosphorylation, phosphorylate, or dephosphorylate PhoB (Hsieh & Wanner, 2010). PhoB is a response regulator for P starved response genes (Kashyap, Botero, Franck, Hassett, & McDermott, 2006). PhoB's phosphorylation state of its receiver domain determines its activity as a transcription factor (Hsieh & Wanner, 2010). There are two different forms *phoB1* and *phoB2*. Their sequences are 93% similar.

pstSCAB-phoU operon. Pst stands for P-specific transport and the system is involved in high affinity P_i transport and turning off the Pho regulon. PhoU is a chaperone-like PhoR/PhoB inhibitory protein (Hsieh & Wanner, 2010). PhoU acts with PhoR to dephosphorylate PhoB-P. *phoU* is in the *pstSCAB-phoU* operon so is translated with *pst* genes (Lamarche et al., 2008). An ABC (ATP-binding cassette) transporter is made of four proteins: PstS, PstC, PstA, and PstB. PstS is a periplasmic P binding protein. PstC and PstA are integral membrane channel proteins. PstB utilized ATP for energy to transport P_i from the periplasm to the cytosol (Lamarche et al., 2008).

As Metabolism Genes

Some bacteria use As in their metabolism. There are two genes operons which are indicated for roles of As metabolism in bacteria. The first is *arr* operon and the second is *arx* operon. They perform reverse reactions, but have sequence similarity which indicates *arx* may be a variant of *arr* (van Lis et al., 2013). The *arr* (As(V) respiratory reductase) operon consists of genes that are involved in As(V) reduction for energy generation. The reduction of As(V) is coupled to organic carbon oxidation to produce energy (Zargar et

al., 2010). The *arx* operon is involved in As(III) oxidation in anaerobic conditions through integration into the electron transport chain in photosynthesis or combined with nitrate respiration (Zargar et al., 2012). The *arx* system is not widespread and only occurs in a limited number of bacteria. It has been suggested that *arxAB* may be ancestral to *aioB* and *arrA* (Oremland et al., 2009).

INTRODUCING THE ArsR REGULATED ARSENIC STIMULON

Introduction

Arsenic (As) tops the substance priority list prepared by the ATSDR and the EPA because of the frequency, toxicity, and potential for human exposure at National Priority List sites (ATSDR, 2016) and the World Health Organization has ranked As among the top ten chemicals that threaten human health (WHO, 2016). Around the world, As contamination hotspots exist from both natural and anthropogenic sources. Once As has entered an environment, it cannot be destroyed but may change forms or solubility through the actions of bacteria or through prevailing environmental chemistry. It can be found in oxidation states of +5 (arsenate), +3 (arsenite), 0 (elemental As), and -3 (arsine) (Kaur et al., 2011), but is typically found in the environment as arsenate [As(V)] and arsenite [As(III)]. Speciation influences toxicity as well as mobility and is determined by redox potential, pH, and microorganism activity (Kang, Heinemann, et al., 2012; Kruger et al., 2013).

Microbes react to As in their environment in several different ways. Some are able to utilize As in a metabolic process, some detoxify As, and some are capable of doing both. Microbe-As interactions are coordinated by various genes which are usually organized into operons. Functions of As related operons include: As(III) extrusions from the cell (*ars* genes), arsenite oxidation (*aio* operon), respiratory As(V) reduction (*arr* operon), and photosynthetic As(III) oxidation (*arx* operon) (Branco et al., 2008). An understanding of the bacterial response to As is essential as many microbial

metal/metalloid-survival techniques involve change of speciation that leads to changes in mobility and toxicity. Thus, microbial activity plays a major role in As biogeochemical cycling (Gadd, 2010).

The As resistance (*ars*) operon provides a survival technique for microbes exposed to As. The *ars* genes are organized into operons that are usually either three or five genes (*arsRBC* or *arsRDABC* respectively) (Butcher & Rawlings, 2002). *arsR* codes for a regulatory protein, *arsB* encodes for an As(III) efflux protein, and *arsC* encodes for a AsV reducing protein. When As(III) enters the cell through aquaglyceroporins, it causes the ArsR protein to be released from the DNA allowing transcription of the *ars* operon and then As(III) is extruded from the cell with the help of ArsB. If As enters the cell as As(V), it is reduced to As(III) with ArsC and then extruded (Rosen, 1999). *arsA* encodes a catalytic subunit of the ArsB efflux pump and *arsD* encodes a metallochaperone that transfers As(III) to the efflux pump for increased efficiency (Branco et al., 2008; Kaur et al., 2011). Additional *ars* genes that may perform functions involved in As resistance include: *arsTX*, *arsO*, *arsP*, *arsH*, *acr3*, *arsI*, and *arsN*. ArsTX is a thioredoxin system that is transcribed along with an *arsRC2* fusion gene (Achour-Rokbani et al., 2010). ArsO is a putative flavin-binding monooxygenase (Wang et al., 2006). ArsP contributes to resistance of organic As compounds by acting as a efflux transporter as studied in *C. jejune* (Shen et al., 2014). ArsH is an organoarsenical oxidase that oxidizes trivalent methylates and aromatic arsenicals (Chen et al., 2015). Acr3 is a protein that functions as a transporter that has a similar function to ArsB (Kang et al., 2015). ArsI controls As methylation and demethylation (Qin et al., 2006; Yoshinaga & Rosen, 2014). ArsN is

closely associated with As resistance genes and has been shown to contribute resistance to sodium As(V) (Chauhan et al., 2009). Although not in the *ars* operon, *arsM* encodes for an arsenic methyltransferase (Qin et al., 2006)

This study focuses on the *arsR* genes. The ArsR protein has regulatory function by binding to the promoter region of the *ars* operon preventing RNA polymerase from accessing the DNA to effect transcription. When As(III) enters the cell and binds to ArsR, protein conformational changes occurs such that ArsR releases from the DNA and thus opening up the operon for expression. ArsR proteins were originally believed to have solely repressor activity. However, ArsR proteins have recently been shown to have activator function as well (Kang, 2016).

Agrobacterium tumefaciens strain 5A has been used widely as a model organism for studying As-microbe interactions. This bacterium has two *ars* loci with two *arsR* genes at each locus. These four *arsR* genes are known as *arsR1*, *arsR2*, *arsR3*, and *arsR4*. *arsR1* and *arsR4* are similar in their sequences (93% identity and 96% similarity). *arsR2* and *arsR3* are more closely related (78% identity and 86% similarity) (Kang et al., 2016). Previous studies have explored the importance of *ars* gene reiteration and this study will lead to a deeper understanding of the *arsR* reiteration through transcriptome analysis (Ordonez, Letek, Valbuena, Gil, & Mateos, 2005; Paez-Espino, Durante-Rodriguez, & de Lorenzo, 2015).

The *arsR* genes have been shown to control the expression of other genes in the *ars* operon as well as select genes outside of the operon (Kang et al., 2015; Rosen, 1999, 2002). ArsR1 has influence over nearby the *phoB-1* and *pstS-1* genes that are involved in

the cell's response to phosphorus stress (Kang, Bothner, et al., 2012). *ArsR2* and *ArsR4* are involved with positive regulation of *aioAB* genes that encode arsenite oxidase (Kang, Heinemann, et al., 2012). Exploration of specific genes controlled by *ArsR* proteins outside of the *ars* operon has been limited.

This study looked at *ArsR* regulatory activity on a bacterial genome-wide scale. Differential gene expression analysis was performed on *arsR* mutants in relation to the wild type strain in order to assess their regulatory roles. Affected genes were grouped into functions to determine the role of each of the *ArsR* proteins in the bacterial cell. This study contributes to a more complete understanding of how microbial gene expression, bacteria reactions to environmental toxins and biogeochemical cycling are influenced by As in the environment.

Methods and Materials

Reporter Constructs and Deletion Mutants

Previously constructed deletion mutations and *lacZ* reporter constructs were used for this study (Table 1). In-frame deletion mutations for *arsR1*, *arsR2*, *arsR3*, and *arsR4* were constructed with the use of crossover PCR as described (Link, Phillips, & Church, 1997) and selection was conducted using the levan-sucrase resistance selection strategy (Summers, Denton, & McDermott, 1999). *lacZ* transcriptional reporter constructs for *arsR1*, *arsR2*, and *arsR4* were created by PCR amplifying the promoter region and directionally cloning them into the multicloning site of pLSP-KT2*lacZ* containing a promoterless *lacZ* coding region (Kang et al., 2016). *arsR3* has been previously shown to

be nonresponsive to As(III) level and may be regulated in conjunction with the upstream *lysR* gene, thus a construct was not created (Kang, 2016).

Table 1. Bacterial Strains and constructs used in this study.

Strains	Relevant markers and characteristics	Reference or Source
5A	Wild type, soil isolate, As(III) oxidizer	Lab stock
$\Delta arsR1$ mutant	<i>arsR1</i> gene deletion mutant	Kang et al. 2016
$\Delta arsR2$ mutant	<i>arsR2</i> gene deletion mutant	Kang et al. 2016
$\Delta arsR3$ mutant	<i>arsR3</i> gene deletion mutant	Kang et al. 2016
$\Delta arsR4$ mutant	<i>arsR4</i> gene deletion mutant	Kang et al. 2016
Constructs		
<i>lacZ::arsR1</i>	<i>ParsR1-lacZ fusion</i>	Kang et al. 2016
<i>lacZ::arsR2</i>	<i>ParsR2-lacZ fusion</i>	Kang et al. 2016
<i>lacZ::arsR3</i>	<i>ParsR3-lacZ fusion</i>	Kang et al. 2016

Bacterial Growth Conditions

A. tumefaciens strains were maintained on minimal mannitol medium (MMNH4) (Somerville & Kahn, 1983) plates that contained 500µg/ml kanamycin for *lacZ* reporter construct maintenance. Cultures were grown and incubated in liquid minimal mannitol medium with low iron (10 fold less) at 30°C in waterbath incubators with aeration by shaking. Reducing Fe^{3+} content for the short term induction and RNA collection experiments did not constitute an iron starvation condition, but was found to be necessary to accommodate RNA yields. As(III) was added at the start of induction periods as indicated. Growth of bacterial cultures was monitored using measurements of culture optical density by a SpectaMax microtiter plate reader (Molecular Devices, California).

arsR Reporter Assays

β -galactosidase reporter constructs for *arsR1*, *arsR2* and *arsR4* in the *A. tumefaciens* wild type strain 5A were used to determine a reasonable As(III) concentration and exposure time for induction. Prior efforts implied that an *arsR3::lacZ* construct does not upregulate in response to As(III) and RT-PCR assays failed to show evidence that As(III) controlled its expression (Kang et al. 2016). Consequently, no reporter assays were conducted for this gene. Initial assays examined a range of As(III) concentrations for a standardized induction period of 60 min. Cells were grown to OD₅₉₅ 0.2. As(III) was added with concentrations of 0, 5, 10, 20, 50, 75, and 100 μ M for the induction period. Subsequent reporter assays then examined reporter expression at these As(III) concentrations as a function of time. Each construct was run with its respective As(III) concentration every 10 minutes for 60 minutes to determine an appropriate incubation period.

RNA Extraction

Overnight cultures were diluted to OD₅₉₅ = 0.1 and allowed to grow until density reached OD₅₉₅ = 0.2 to ensure cells were in growth phase when As(III) was then added. Each treatment (5ml volume for treatments without As(III), and 10 ml volume for treatments with As(III)) was incubated at 30°C in a waterbath incubator with aeration by shaking with the appropriate As(III) concentration for 40 minutes. Cells were collected by centrifugation (8,000xg for 5 min at 4°C), suspended in Qiagen RNeasy Protect to protect cellular RNA and then centrifuged at 5,000xg at 4°C for 10 min. The cell pellet was suspended with 200 μ l TE buffer containing 1 mg/ml lysozyme and cells were lysed by

pipetting for 5 min. RNA was then extracted using the Qiagen RNeasy Minikit following the manufacturer protocol, including the addition of 2.5 ml RLT buffer in Step 3 as per recommendation to account for quantity of cells being used. DNAsing was done with Qiagen DNase kit. RNA concentrations were measured with the SpectaMax microtiter plate reader. The RNA quality and purity was determined with an Agilent 2100 Bioanalyzer (Agilent Technologies). RNA was stored at -80°C until sequencing.

RNA Sequencing

Sequencing was completed at Brigham Young University (Provo, UT). Briefly, rRNA was removed using Illumina Ribo-Zero rRNA removal kit. The RNA was fragmented into 50 bp segments and reverse transcribed into first strand cDNA using random primers and reverse transcriptase. Second strand cDNA synthesis was then completed using DNA Polymerase I and RNase H. Next, the cDNA went through end repair process, the 3' ends are adenylated, and sequence adapters were ligated to the cDNA fragments. PCR was used to purify and enrich the cDNA to create the final library. Our samples were normalized prior to pooling using qPCR. Sequencing was done using high throughput Illumina TruSeq.

Sequence Analysis

BowTie was used to remove ribosomal RNA sequences. Quality control was done with the use of FASTQC, trimming of adaptors was completed with Trimmomatic and mRNA quantification was completed using Kallisto. Sleuth was used to perform differential gene expression analysis. Transcript names from Sleuth were input to Uniprot

for gene annotation purposes and any unidentified proteins had their sequences searched in BLAST. The significant gene influence threshold was determined by the absolute value of the fold change being greater than two and having a q value equal to or less than 0.05.

Results

arsR reporter Assays

The relative position of the four different *arsR* genes considered in this study are shown in Fig. 6. β -galactosidase reporters for *arsR1*, *arsR2* and *arsR4* in the *A. tumefaciens* wild type strain 5A were used to determine a reasonable As(III) concentration and exposure time for induction of the encoded proteins that would then translate to near maximum regulatory activity exerted by each ArsR. Optimal reporter activity within a constrained period was viewed as a reasonable proxy for estimating best conditions for subsequent experiments that would focus on RNASeq analysis of the different *arsR* mutants (below). By limiting the As(III) exposure period, the goal was to induce an As(III) transcriptional response, but reduce or avoid potential downstream cascade effects that could derive from other transcriptional regulators controlled by these ArsRs that would elicit a second wave of transcriptional responses.

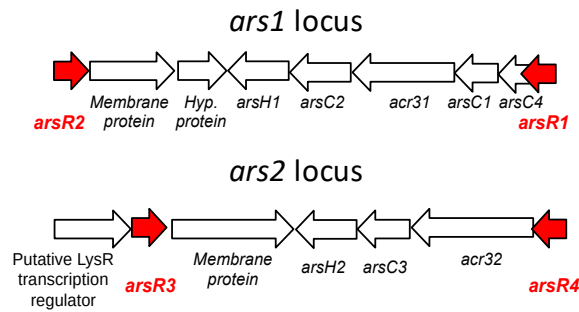


Figure 6. Organization of the *arsR1* and *arsR2* loci. The *arsR* genes that are the focus of this study are highlighted in res. (Modified from Kang, 2016).

Initial assays examined a range of As(III) concentrations for a standardized induction period of 60 min (Fig. 7A). Optimum As(III) concentrations for maximum (or nearly so) were 100 μ M, 50 μ M, and 75 μ M for the *arsR1::lacZ*, *arsR2::lacZ* and *arsR4::lacZ* reporters, respectively. Maximum expression levels varied substantially, with *arsR4::lacZ* being the most robust. Prior efforts showed that an *arsR3::lacZ* does not regulate in response to As(III) and RT-PCR assays suggested low or poor induction in response to As(III) (Kang et al. 2016). Consequently, no reporter assays were conducted for this gene. Subsequent reporter assays then examined reporter expression at these As(III) concentrations to determine optimal induction times (Fig. 7B). It was found that expression levels after 40 min. were at or near maximum and that extending incubations times would not appreciably enhance the sensitivity of gene expression levels sought in the RNASeq work.

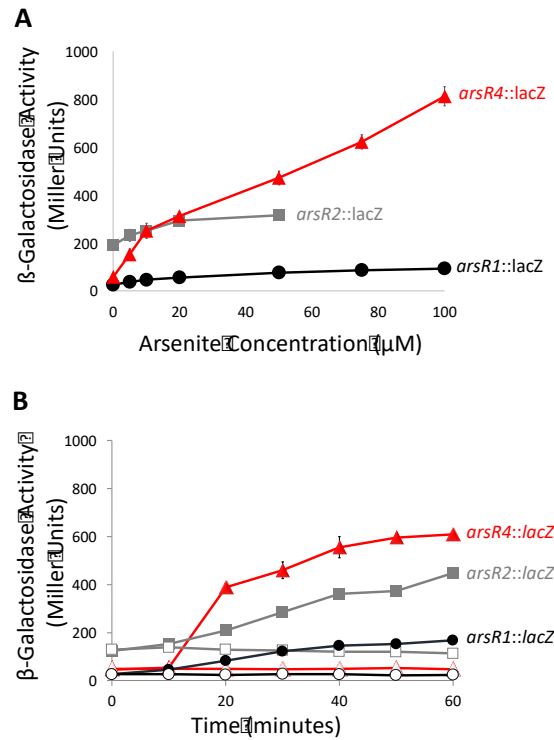


Figure 7. Reporter gene assays monitoring expression of *arsR1*, *arsR2*, and *arsR4* as a function of As(III) exposure and time. (A) Reporter gene assay monitoring expression as a function of As(III) exposure. (B) Reporter gene assay monitoring expression as a function of time. Filled symbols, +As(III); open symbols, -As(III) controls. Where visible, error bars indicate ± 1 standard deviation

Gene Expression Changes Related to As(III) Exposure

Based on the above reporter activity assays, initial experiments considered the response of the wild type *A. tumefaciens* strain exposed to 0, 50 μM, 75 μM, and 100 μM As(III) for a 40 minute incubation period. Changes were considered significant if the absolute value of the fold change was ≥ 2.0 and the q-value was ≤ 0.05 . In total, 138 genes were significantly upregulated and 111 genes significantly downregulated (Table 2). As expected based on prior research (Kang et al. 2012; 2016), several genes involved in As resistance and phosphate acquisition/metabolism showed increased expression.

Other upregulated functions included iron homeostasis, five involved with DNA replication, membrane proteins, four coding for ABC transporters, protein processing, and three coding for sugar transporters. Ten iron homeostasis genes were also downregulated, along with seven encoding some element of chemotaxis, membrane proteins, β -lactam resistance (five genes), transcription regulation (four genes), molybdenum transport (four genes), and copper tolerance (six genes). Transcriptional responses to the different As(III) concentrations varied. For genes that were upregulated by the lowest As(III) level, transcription was often found to increase with increased exposure to As(III). Examples include all of the *ars* genes coding for As resistance and most of the phosphate stress response genes (Table 2), although increases for most of the latter were more subtle. Many other functions upregulated at only the highest As(III) exposure (e.g., iron transport, DNA replication, transporters) as was also the case for many of the downregulated functions such as genes involved in chemotaxis and other iron homeostasis (Table 2). One sugar transporter operon upregulated at the lower As(III) levels but then was repressed at the highest As(III) (Table 2).

Table 2. Significant gene expression changes in wild type *A. tumefaciens* 5A as a function of As(III) exposure. Cells were exposed to As(III) at three different concentrations (50µM, 75µM, and 100µM) for a 40 minute incubation period. Expression ratios were calculated as gene expression levels as + As(III) expression divided by As(III) expression. Only functions with at least two related genes are included in this table.

As(III) treatment			Gene Names	Gene Annotation/Function
50μM	75μM	100μM		
Increased Expression				
Arsenic Response				
		2.06	AT5A_08115	ArsR family protein
		4.39	AT5A_22226	ArsR3 transcriptional regulator
23.34	35.30	92.71	AT5A_22236	ArsH2 Arsenical resistance protein
31.18	45.05	124.12	AT5A_22241	ArsC3 Arsenate reductase
44.73	63.87	178.82	AT5A_22246	Acr3-2 Arsenite efflux pump protein
34.68	51.90	135.53	AT5A_22251	ArsR4 family transcriptional regulator
2.48	3.19	18.13	AT5A_25245	ArsR family transcriptional regulator
	2.24		AT5A_25560	AioA Arsenite oxidase large subunit
11.75	13.73	16.24	AT5A_25620	ArsC Arsenate reductase
43.73	52.44	60.75	AT5A_25645	ArsR1 transcriptional regulator
35.25	43.85	61.26	AT5A_25650	ArsC4 Arsenate reductase
66.65	89.51	150.39	AT5A_25655	ArsC1 Arsenate reductase
60.49	82.76	126.04	AT5A_25660	Acr3-1 Arsenite efflux pump protein
53.89	74.60	122.32	AT5A_25665	ArsC2 Arsenate reductase
50.27	70.20	104.13	AT5A_25670	ArsH1 Arsenical resistance protein
	2.14	6.97	AT5A_25685	ArsR2 transcriptional regulator
Phosphorous Acquisition/Metabolism				
		2.25	AT5A_11977	Phosphate ABC transporter
9.02	9.80	14.66	AT5A_25585	PhoB Phosphate regulon regulatory protein
8.60	10.06	14.08	AT5A_25590	PstS1 Phosphate binding
2.95	3.68	3.87	AT5A_25595	PstC1 Phosphate transport system permease
6.53	8.31	8.20	AT5A_25600	PstA1 Phosphate transport permease protein
4.25	5.24	6.46	AT5A_25605	PstB1 Phosphate import ATP-binding protein
3.91	4.77	5.46	AT5A_25610	PhoU1 Phosphate transport system protein
5.67	7.12	7.90	AT5A_25615	Phosphate regulon regulatory protein
7.72	7.90	15.56	AT5A_25625	Phosphonate ABC transporter
6.63	6.92	8.65	AT5A_25630	PhnC Phosphonates import ATP-binding
3.88	4.24	4.78	AT5A_25635	Phosphonate ABC transporter, inner membrane
3.09	2.79	3.43	AT5A_25640	Phosphonate ABC transporter, inner membrane
Iron Homeostasis				
		5.03	AT5A_00040	Periplasmic chelated iron-binding protein
		3.43	AT5A_00050	Iron ABC transporter ATP-binding protein
		2.41	AT5A_10100	FbpC Fe(3+) ions import ATP-binding protein
		2.14	AT5A_20071	Iron ABC transporter substrate-binding protein
2.01	1.99		AT5A_23725	Iron ABC transporter permease

Table 2. Continued.

As(III) treatment				
50μM	75μM	100μM	Gene Names	Gene Annotation
Increased Expression				
<u>DNA Replication</u>				
		3.29	AT5A_10287	Glutaredoxin-like protein
		3.56	AT5A_10297	Ribonucleoside-diphosphate reductase
		3.19	AT5A_10302	NrdF Ribonucleoside-diphosphate reductase
		2.07	AT5A_14197	Ribonucleotide-diphosphate reductase
		2.03	AT5A_14867	DNA polymerase III subunit beta
<u>Membrane Protein</u>				
		2.26	AT5A_10105	Binding-protein-dependent transport systems
		2.17	AT5A_20431	Extracellular solute-binding protein family 1
8.77	13.70	28.38	AT5A_22231	Major facilitator transporter
22.49	31.91	47.22	AT5A_25675	UPF0721 transmembrane protein
2.11	2.31	5.02	AT5A_25680	Major facilitator transporter
<u>ABC Transporter</u>				
		3.87	AT5A_12602	Alpha-glucoside ABC transporter
		2.85	AT5A_12607	ABC transporter transmembrane protein
		2.67	AT5A_12612	ABC transporter transmembrane protein
		2.00	AT5A_24165	Proline/glycine/betaine substrate binding
<u>Protein Processing</u>				
		2.22	AT5A_19831	5-methyltetrahydropteroyltriglutamate
3.72	2.04		AT5A_26335	GroES 10 kDa chaperonin
3.32			AT5A_26340	GroEL 60 kDa chaperonin
<u>Sugar Transport</u>				
	2.27		AT5A_00215	Ribose ABC transporter substrate binding
	2.00		AT5A_00220	Ribose ABC transporter nucleotide binding
		2.30	AT5A_01015	Monosaccharide-transporting ATPase
<u>Acetylation of Amine</u>				
		4.92	AT5A_04340	GCN5-related N-acetyltransferase
2.13	2.36	8.57	AT5A_25235	GCN5-related N-acetyltransferase
<u>Amino Acid Metabolism</u>				
2.03			AT5A_01565	2-oxoisovalerate dehydrogenase subunit alpha
		9.63	AT5A_22851	Aspartate racemase
<u>Amino Acid Transport</u>				
2.03	2.16		AT5A_16886	Amino acid ABC transporter permease
		2.44	AT5A_23385	Oligopeptide ABC transporter protein
<u>Carbohydrate Metabolism</u>				
		3.22	AT5A_22696	UTP--glucose-1-phosphate uridylyltransferase
		2.09	AT5A_22721	Endo-beta-1,3-1,4 glucanase
<u>Polysaccharide Biosynthesis</u>				
		2.02	AT5A_00770	ExoY Exopolysaccharide production protein
		2.00	AT5A_01935	Endo-1,3-1,4-beta-glycanase

Table 2. Continued.

As(III) treatment				
50µM	75µM	100µM	Gene Names	Gene Annotation
Increased Expression				
<u>Glutamate Synthesis</u>				
		2.41	AT5A_02785	GltD Glutamate synthase subunit beta
		2.34	AT5A_02795	Glutamate synthase, large subunit
<u>Cobalamin Biosynthesis</u>				
		2.50	AT5A_04320	Uncharacterized protein
	2.04	2.03	AT5A_04325	Uncharacterized protein
<u>Transcription Regulator</u>				
2.54	2.19		AT5A_19361	MtIR Transcriptional activator
		2.09	AT5A_20676	TetR family transcriptional regulator
Decreased Expression				
<u>Iron Homeostasis</u>				
		-2.80	AT5A_01140	Iron ABC transporter nucleotide binding
		-3.85	AT5A_09690	HmuV Hemin import ATP-binding protein
		-3.31	AT5A_11337	Iron-regulated protein
		-2.70	AT5A_11917	Iron ABC transporter nucleotide-binding
		-2.09	AT5A_11927	Iron ABC transporter transmembrane protein
		-2.33	AT5A_15846	Iron ABC transporter, membrane spanning
		-2.24	AT5A_16421	Ferrichrome ABC transporter
		-2.60	AT5A_16431	Ferrichrome transport system permease
		-2.42	AT5A_16631	Fe3+ siderophore ABC transporter permease
		-2.57	AT5A_23515	Iron ABC transporter permease
<u>Chemotaxis</u>				
		-2.69	AT5A_10140	CheB Chemotaxis response regulator protein
		-2.12	AT5A_10150	CheW Chemotaxis protein
		-2.15	AT5A_10155	Methyl-accepting chemotaxis protein
		-2.23	AT5A_10160	CheW Chemotaxis protein
		-2.54	AT5A_10165	Chemotaxis protein histidine kinase
		-2.23	AT5A_10170	Chemotaxis receiver protein
		-2.07	AT5A_12222	Methyl-accepting chemotaxis protein
<u>Membrane Protein</u>				
		-2.50	AT5A_02375	Uncharacterized protein
	-3.46	-2.60	AT5A_08840	Uncharacterized protein
		-2.43	AT5A_08260	PA-phosphatase related phosphoesterase
-2.66	-2.39		AT5A_09745	Putative tricarboxylic transport membrane
		-2.39	AT5A_09775	ABC transporter transmembrane protein
	-2.12		AT5A_22911	Protein YBGT-like protein

Table 2. Continued.

As(III) treatment			Gene Names	Gene Annotation
50μM	75μM	100μM		
Decreased Expression				
β-lactam Resistance				
-2.28	-2.65	-2.87	AT5A_00790	Uncharacterized protein
	-2.23	-2.95	AT5A_00795	Uncharacterized protein
	-2.03	-2.59	AT5A_15482	Beta-lactamase
	-1.99	-2.37	AT5A_05015	Soluble lytic transglycosylase
	-2.36		AT5A_12757	Metallo-beta-lactamase superfamily protein
Transcription Regulation				
-2.58	-2.15		AT5A_22886	Putative transcriptional regulator protein
-2.04			AT5A_23980	RbsR Transcriptional repressor
		-2.28	AT5A_02905	AsnC Transcriptional regulator, family protein
		-2.18	AT5A_15701	RpiR family transcriptional regulator
Molybdenum Transport				
	-2.20	-5.17	AT5A_19111	Molybdenum ABC transporter
	-2.18	-4.59	AT5A_19106	ModB Molybdate ABC transporter permease
	-2.02	-3.85	AT5A_19101	ModC Molybdenum import ATP-binding
		-2.52	AT5A_19096	Molybdenum transport protein modE
Copper Tolerance				
-5.93	-5.79	-13.88	AT5A_22416	Uncharacterized protein
-2.86		-3.66	AT5A_04590	Transcriptional regulator, MerR family protein
-2.15		-3.09	AT5A_05820	Uncharacterized protein
		-3.85	AT5A_22441	Uncharacterized protein
-5.50	-5.05	-13.55	AT5A_22421	Copper tolerance protein
-2.26	-2.44	-8.07	AT5A_22431	Copper tolerance protein
ABC transporter				
-2.15			AT5A_22891	ABC transporter nucleotide binding/ATPase
		-2.05	AT5A_09780	ABC transporter nucleotide-binding protein
ATPase Activity				
-4.17	-2.78		AT5A_16046	ABC transporter nucleotide binding/ATPase
-2.19		-4.18	AT5A_05780	Heavy metal-transporting ATPase
Benzoate Degradation				
-6.37	-4.16		AT5A_16121	3-oxoadipate enol-lactonase
-4.88	-3.54		AT5A_16116	Gamma carboxymuconolactone decarboxylase
Cold Shock Response				
		-5.90	AT5A_21481	Cold shock protein
		-4.79	AT5A_27266	Cold shock protein
Drug Resistance				
-2.02	-2.16		AT5A_00505	AcrB/AcrD/AcrF family protein
		-2.07	AT5A_07475	Acriflavin resistance protein
Respiration				
	-2.22		AT5A_13317	FixQ Cytochrome-c oxidase chain
	-2.09		AT5A_04310	Cytochrome C-556

Table 2. Continued.

As(III) treatment				
50μM	75μM	100μM	Gene Names	Gene Annotation
Decreased Expression				
<u>Glycosyl Transfer</u>				
		-2.84	AT5A_03370	Glycosyl transferase family protein
		-2.46	AT5A_01915	Glycosyltransferase
<u>Queuosine Biosynthesis, Purine Metabolism</u>				
		-2.56	AT5A_00865	QueC 7-cyano-7-deazaguanine synthase
		-2.05	AT5A_00855	QueE 7-carboxy-7-deazaguanine synthase
<u>Oxoreductase Activity</u>				
		-2.60	AT5A_05785	Naphthalene 1,2-dioxygenase
		-2.31	AT5A_11332	Thiol oxidoreductase
As(III) concentration dependent				
<u>Sugar Transport</u>				
2.20	2.47	-17.22	AT5A_19386	Sugar ABC transporter ATP-binding protein
7.16	7.82	-5.52	AT5A_19366	Sugar ABC transporter periplasmic sugar-binding
4.18	4.61	-9.07	AT5A_19376	Multiple sugar transport system permease protein
5.66	6.31	-6.08	AT5A_19371	Sugar ABC transporter permease

Evaluating Changes in Gene Expression Levels of the *arsR* Deletion Mutants

For RNASeq analysis of genome-wide regulatory control by each ArsR, the same deletion mutants previously described by Kang et al. (2016) were used in +/- As(III) experiments using the optimized As(III) concentrations and exposure times described above (Fig. 2). Comparing transcriptional patterns for each mutant against those observed in the wild type strain would identify those genes controlled by each ArsR. Gene expression was considered significantly changed if the absolute value of the fold change was ≥ 2.0 and the q-value was ≤ 0.05 .

Based on current paradigms of how ArsR-type repressors control gene expression, removing the proteins (i.e., deletion mutation) should result in constitutive expression of genes that are normally repressed regardless of presence or absence of the inducing ligand. In this case, in the absence of As(III), expression of ArsR repressed genes should

be significantly increased in an $\Delta arsR$ mutant relative to the wild type cell, and thus an increased transcript ratio would indicate the gene is normally repressed by the specific ArsR, whereas decreased expression suggests the gene is somehow activated by the respective ArsR. The volcano plots in Fig. 8 offer a visual relative assessment of total gene expression patterns for each mutant as compared the wild type. In each case, there were both positive and, surprisingly, negative changes in gene transcript levels. For example, expression of 54 genes were significantly increased in the $\Delta arsR1$ mutant, indicating these genes are normally repressed by ArsR1. In contrast, 50 genes were significantly downregulated in the $\Delta arsR1$ mutant, implying that these genes are normally activated either directly or indirectly by ArsR1. Overall, it appears that ArsR2 exerts the greatest influence over cellular gene expression patterns (233 genes total), whereas ArsR3 influences the fewest (63 genes) (Fig. 8). A more in-depth discussion of transcriptional patterns controlled by each ArsR follows below.

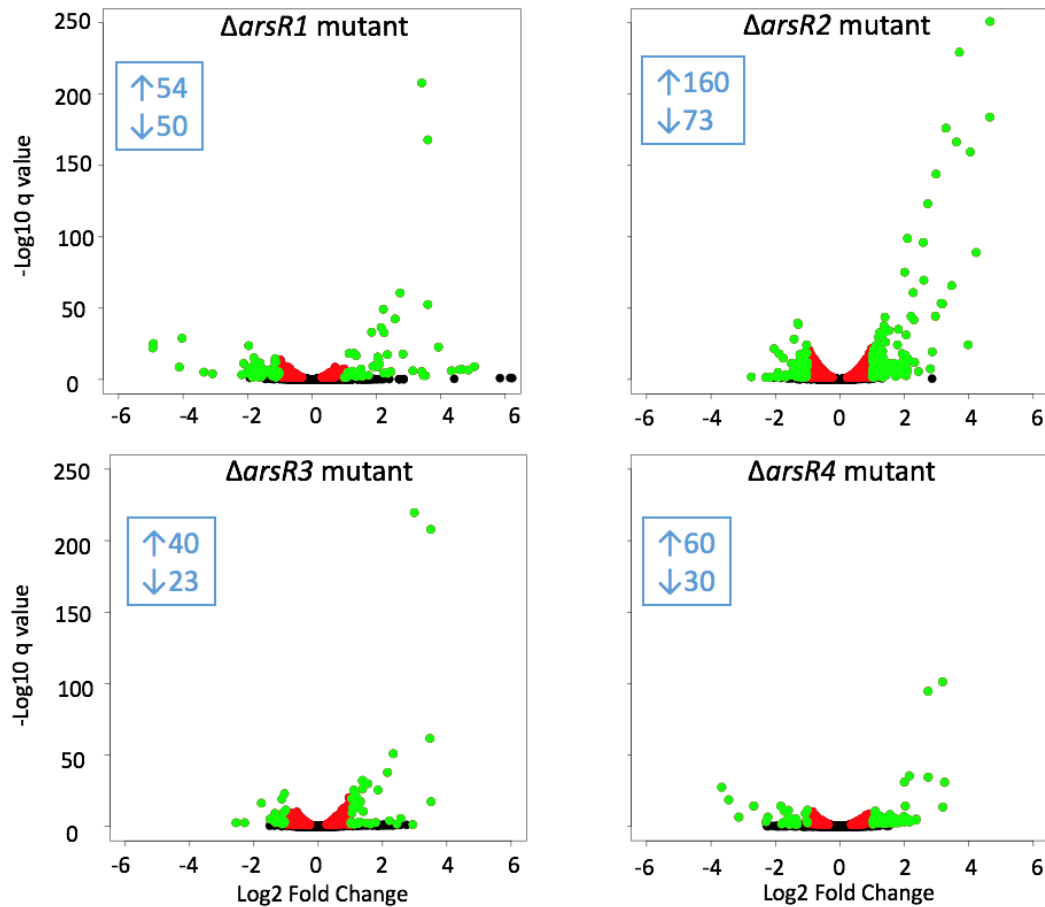


Figure 8. Volcano plots illustrating the influence of each *ArsR* on genome-wide transcription in the absence of As(III). In each case, expression changes are calculated as the ratio of the mutant to the wild type. Red colored dots indicate genes that were significantly changed (q value ≤ 0.05), but less than two-fold. Green dots indicate genes that were significantly changed (q value ≤ 0.05) and had a fold change $\geq \pm 2.0$. Blue boxes in the upper left hand corner of each plot indicate the number of genes with increased expression (would be repressed by the respective *ArsR* in wild type in the absence of As(III)) and number of genes with decreased expressions (presumably activated by the respective *ArsR* in the wild type).

To describe these changes in a functional sense, genes that were significantly influenced in any of the *ArsR* mutants as determined above were grouped into their general functions (Table 3). For this specific analysis, each gene was counted only once, even if it was regulated by more than one *ArsR* protein. Only functions with at least two

significantly changed related genes are listed with their function; all functions with a single related gene are included in the “other” category. In the absence of As(III), genes that were overexpressed (i.e. up-regulated) in the mutant relative to the wild type strain were interpreted to be normally repressed by these ArsRs, whereas genes that were under expressed in the mutants were interpreted to be activated (directly or indirectly) by these regulators. Overall, there were 348 genes significantly influenced by ArsR1, ArsR2, ArsR3, and/or ArsR4. A total of 228 genes represented functions spanning across all aspects of cell physiology, whereas 120 genes that we classify as being activated by these ArsRs again represented fewer cell functions, but nevertheless still significant.

Table 3. Summary of functions influenced by ArsR proteins. Functions were determined by automated gene annotation and function categories are specifically listed if the function is represented by at least two genes. Any function with only one gene was included in the 'Other' category. The 'Activated/Repressed' column includes genes activated by one/some ArsRs and repressed by another ArsR.

Function	Regulation Pattern		Activated/ Repressed	Total
	Activated	Repressed		
Amine Acetylation		2		
Amino Acid Transport and Metabolism	10	10		
Arsenic Exposure Response	1	10	1	
Benzoate Degradation	3			
Biotin Synthesis	2	3		
Carbon Metabolism	1	9		
Chemotaxis	5	1		
Cobalamin Biosynthesis		4		
Cold Shock Response	2			
Copper Tolerance	6	1		
DNA Replication		4		
Glycosyl Transfer	3			
Heat Shock Response		7		
Hydrolase Activity	1	2		
Iron Homeostasis	1	7	2	
Lipid A Synthesis	1	1		
Lysine Biosynthesis		2		
Membrane Protein	8	13	1	
Monooxygenase		2		
Nitrate Reduction		2		
Respiration	2	11		
Nitrogen Transport/Regulation	5			
Organic Acid Transport		2		
Oxoreductase Activity	2	5		
Phosphorus Acquisition/Metabolism		12		
Pilus Assembly	2			
Protein Modification		3		
Queuosine Biosynthesis, Purine Metabolism	3			
Ribosomal Protein	2			
RNA Polymerase Binding		2		
Signal Transduction	3	3		
Sugar Transport		20		
Sulfate Transport	5			
Transcription Regulator	3	4		
Uncharacterized Proteins	23	38		
Universal Stress Response		5		
β -lactam Antibiotic Resistance	6	1		
Other	20	42		
Total	120	228	4	352

Genes Potentially Co-Regulated by All Four ArsR Proteins. In the absence of As(III), transcriptional patterns of 13 genes were found to be significantly altered in all four mutants relative to wild type (Table 4). Ten genes showed increased expression in the mutants, indicating they may be repressed (directly or indirectly) by each of the ArsR proteins in the wild type. Functions affected relate to sugar transport, RNA polymerase binding, organo-phosphate metabolism, carbon metabolism, nitrogen metabolism, and amino acid transport. Three genes had decreased expression in the mutants, indicating possible activation. Relative levels of change differed between the different mutants; e.g., fold change in transcription of a putative multicopper oxidase was activated all four ArsRs, but ranged from -17.4 in the *arsR1* mutant to -2.5 in the *arsR3* mutant, showing different apparent levels of regulatory influence over this gene. Expression ratios of genes belonging to an acid phosphatase super family and encoding a multicopper oxidase are consistent with them being activated by all four ArsR proteins. Surprisingly, expression patterns of the arsenite oxidase small subunit suggested it may be activated by each ArsR to the same degree.

Table 4. Genes exhibiting expression patterns suggesting they are influenced directly or indirectly by all of the ArsR proteins. This table includes genes that were significantly changed by ArsR1, ArsR2, ArsR3, and ArsR4. Gene expression ratios were calculated as gene expression levels in the *arsR* mutant divided by the levels in the wild type.

<i>ΔarsR1</i>	<i>ΔarsR2</i>	<i>ΔarsR3</i>	<i>ΔarsR4</i>	Genome identifier	Gene Annotation/Function
Repressed					
<u>Sugar Transport</u>					
5.47	7.00	5.92	5.11	AT5A_19366	Sugar ABC Transporter Periplasmic Protein
4.67	5.42	4.65	4.03	AT5A_19371	Sugar ABC Transporter Permease
3.38	3.75	3.45	3.04	AT5A_19376	Multiple Sugar Transport System Permease
<u>RNA Polymerase Binding</u>					
12.10	9.78	11.33	9.11	AT5A_20191	Nucleoside Diphosphate Kinase Regulator
4.43	4.91	4.45	4.01	AT5A_20201	Nucleoside Diphosphate Kinase Regulator
<u>Miscellaneous</u>					
15.20	15.77	11.40	9.17	AT5A_20166	Hypothetical protein
12.10	7.80	11.12	9.51	AT5A_20186	Permease Protein
10.61	7.91	7.95	6.67	AT5A_20181	Glutamate Dehydrogenase
6.65	4.66	5.04	4.46	AT5A_20176	Hypothetical Protein
4.72	4.16	3.63	4.02	AT5A_20171	NUDIX Hydrolase
Activated					
<u>Miscellaneous</u>					
-17.35	-4.48	-2.54	-4.89	AT5A_22426	Multicopper Oxidase
-16.32	-3.73	-2.04	-6.37	AT5A_22411	Acid Phosphatase sSuperfamily
-3.06	-3.70	-3.40	-3.55	AT5A_25565	Arsenite Oxidase Small Subunit

Genes Regulated by ArsR1 Only. The next layer of analysis sought to identify those genes uniquely controlled by ArsR1 (Table 5). Again, comparing transcript levels of the *ΔarsR1* mutant against the wild type in the absence of As(III) revealed both increased and decreased expression ratios, inferring repression and activation, respectively. Expression changes were observed for 104 genes in the *ΔarsR1* mutant, but of these a subset of 36 were unique to ArsR1; i.e., expression changes in these genes were not observed in any of the three other mutants. In the absence of As(III) treatment, upregulation in the mutant relative to wild type indicates the transcription of the gene is no longer constitutively repressed.

Table 5. Gene expression significantly influenced by ArsR1 only. This table includes genes that are only influenced by ArsR1 (not ArsR2, ArsR3, or ArsR4). Expression ratios were calculated as gene expression levels in the arsR1 mutant divided by those in the wild type.

Fold Change	Gene Name	Gene Annotation/Function
Genes Repressed		
<u>Arsenic Response</u>		
4.45	AT5A_22236	ArsH2 Arsenical resistance protein
5.19	AT5A_22241	ArsC3 Arsenate reductase
4.83	AT5A_22251	ArsR4 family transcriptional regulator
5.75	AT5A_25620	ArsC Arsenate reductase
4.52	AT5A_25645	ArsR1 Family transcriptional regulator
14.43	AT5A_25650	ArsC4 Arsenate reductase
11.50	AT5A_25655	ArsC1 Arsenate reductase
10.77	AT5A_25660	Acr3-1 Arsenite efflux pump
8.74	AT5A_25670	ArsH1 Arsenical resistance protein
<u>Phosphorus Acquisition/Metabolism</u>		
5.80	AT5A_25585	PhoB1 Phosphate regulon transcriptional regulatory
5.16	AT5A_25590	PstS1 Phosphate binding
2.03	AT5A_25595	PstC1 Phosphate transport system permease
3.37	AT5A_25600	PstA1 Phosphate transport system permease
3.05	AT5A_25605	PstB1 Phosphate import ATP-binding protein
2.35	AT5A_25610	PhoU1 Phosphate-specific transport system accessory
3.30	AT5A_25615	Phosphate regulon transcriptional regulatory protein
5.20	AT5A_25625	Phosphonate ABC transporter, periplasmic phosphonate-binding
4.34	AT5A_25630	PhnC Phosphonates import ATP-binding
2.64	AT5A_25635	Phosphonate ABC transporter, inner membrane subunit
2.02	AT5A_25640	Phosphonate ABC transporter, inner membrane subunit
<u>Miscellaneous</u>		
4.75	AT5A_05975	Fe III dicitrate ABC transporter, permease
2.21	AT5A_09865	Aspartate-semialdehyde dehydrogenase
Genes Activated		
<u>Oxioreductase Activity</u>		
-2.23	AT5A_01335	Putative flavin reductase
-2.54	AT5A_05785	Naphthalene 1,2-dioxygenase
<u>Pilus Production</u>		
-2.28	AT5A_13612	TadE family protein
-2.62	AT5A_13617	tad related protein
<u>Nickel Transport</u>		
-2.18	AT5A_05795	NikB Nickel transport system permease protein
-2.11	AT5A_05805	Nickel import ATP-binding protein NikD
<u>Miscellaneous</u>		
-2.39	AT5A_01350	Putative hydrolase
-2.54	AT5A_08835	Permease protein
-2.25	AT5A_15482	Beta-lactamase
-2.04	AT5A_16046	ABC transporter nucleotide binding/ATPase protein
-2.30	AT5A_19626	Glutathione S-transferase domain protein
-2.02	AT5A_22471	8-amino-7-oxononanoate synthase
-3.49	AT5A_22636	Glucose dehydrogenase
-2.14	AT5A_23201	SsuB Aliphatic sulfonates import ATP-binding protein

Most genes involved are physically associated nearby the *arsR1* gene that defines the *ars1* locus (Fig. 9), but the *arsR4* operon in the distal *ars2* locus is also negatively regulated by ArsR1 (Table 5). These observations are consistent with previous work showing that ArsR1 represses itself and *arsR4*, *phoB1* and *pstS1* (Kang et al., 2012). Decreased expression of 18 genes in the Δ *arsR1* mutant indicates that ArsR1 potentially is involved in the activation of these genes. Functions involved in this context include oxidoreductase activity, pilus production, and nickel transport.

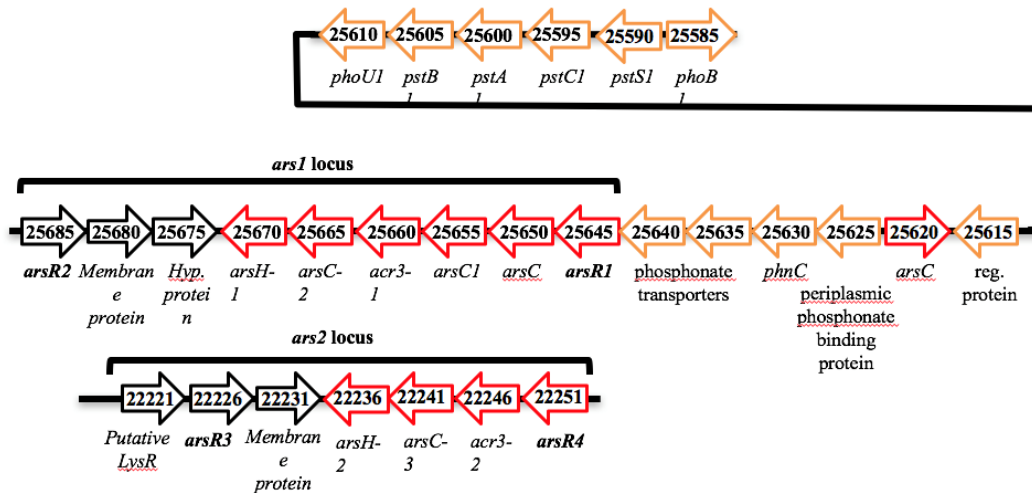


Figure 9. Genes uniquely repressed by ArsR. Arsenic resistance genes are outlined in red and phosphorus transport and metabolism related genes are outlined in orange. Numbers inside the gene identifier in the AT5A genome (can cross-reference with Table 5).

Genes Regulated by ArsR2 Only. A large number of genes exhibited ArsR2-dependent expression patterns; 100 genes showed increased expression indicating they are normally repressed by ArsR2, and 53 were downregulated, indicating they are normally activated (Table 6). Functions repressed include sugar transport, heat shock, oxidoreductase activity, membrane protein genes, non-specified transporters, signal

transduction, biosynthesis of cobalamin and lysine, acetylation of primary amines, and organic acid transport. Importantly, two genes encoding ArsR transcriptional repressors are also repressed by ArsR2, including *arsR3*, and thus demonstrating regulatory interaction between these two ArsR proteins as was observed for ArsR1 with *arsR4* (Table 5). Functions apparently activated by ArsR2 include amino acid transport, chemotaxis, nitrogen transport, sulfate transport, queuosine biosynthesis, purine metabolism, membrane proteins, amino acid biosynthesis, synthesis of ribosomal proteins, glycosyl transfer, and signal transduction.

Table 6. Gene expression significantly influenced by ArsR2 only. Expression ratios were calculated as gene expression levels in the *arsR2* mutant relative to wild type.

Fold Change	Gene Name	Gene Annotation/Function
<u>Genes Repressed</u>		
<u>Sugar Transport</u>		
2.34	AT5A_03235	Putative multiple sugar transport system
2.06	AT5A_03250	Sugar ABC transporter permease
2.39	AT5A_03255	Sugar ABC transporter nucleotide-binding
2.56	AT5A_03260	Sugar ABC transporter ATPase
2.58	AT5A_09935	Sugar ABC transporter substrate-binding
2.28	AT5A_09940	Sugar ABC transporter transmembrane
2.06	AT5A_09945	Sugar ABC transporter transmembrane
2.23	AT5A_09950	Sugar ABC transporter nucleotide-binding
2.36	AT5A_22281	Sugar ABC transporter permease
2.36	AT5A_22286	Sugar ABC transporter substrate-binding
<u>Heat Shock Response</u>		
2.48	AT5A_19336	HtpG Chaperone protein
2.77	AT5A_03215	IbpA Small heat shock protein
2.75	AT5A_09630	RpoH RNA polymerase sigma factor
2.04	AT5A_11772	Small heat shock protein
2.54	AT5A_14392	HspQ Heat shock protein
3.30	AT5A_21091	Small heat shock protein
3.98	AT5A_23306	ClpB Chaperone protein
<u>Oxidoreductase Activity</u>		
3.54	AT5A_04245	Aldo/keto reductase
2.01	AT5A_18901	Oxidoreductase protein
2.15	AT5A_22291	Oxidoreductase
2.25	AT5A_22296	Oxidoreductase
<u>Membrane Protein</u>		
2.16	AT5A_03245	Binding-protein-dependent transport systems
2.05	AT5A_17106	Membrane protein
2.53	AT5A_18551	Membrane protein family DUF 1232
<u>Transport Across Membrane</u>		
2.39	AT5A_16521	Oligopeptide ABC transporter substrate binding protein
2.50	AT5A_20111	Oligopeptide ABC transporter transmembrane protein
2.34	AT5A_20116	Oligopeptide ABC transporter substrate-binding protein
<u>Signal Transduction</u>		
2.12	AT5A_18541	Protein tyrosine phosphatase
4.09	AT5A_22516	Putative adenylate cyclase protein
3.82	AT5A_25240	Protein-tyrosine-phosphatase
<u>Arsenic Response</u>		
2.53	AT5A_22226	ArsR3 family transcriptional regulator
3.27	AT5A_25245	ArsR family transcriptional regulator
<u>Cobalamin Biosynthesis</u>		
2.64	AT5A_17901	CobW Cobalamin biosynthesis protein
2.33	AT5A_17906	CobN Cobaltochelate subunit
<u>Lysine Biosynthesis</u>		
4.33	AT5A_09925	Dihydrodipicolinate synthase
2.06	AT5A_20131	Dihydrodipicolinate synthase
<u>Acetylation of Amine</u>		
3.19	AT5A_04340	GCN5-related N-acetyltransferase
4.99	AT5A_25235	GCN5-related N-acetyltransferase

Table 6. Continued.

Fold Change	Gene Name	Gene Annotation/Function
Genes Repressed		
Organic Acid Transporter		
2.00	AT5A_00375	DctP/TAP dicarboxylate transporter subunit
2.25	AT5A_00380	
Miscellaneous		
2.09	AT5A_02225	GloB Hydroxyacylglutathione hydrolase
2.44	AT5A_03220	Na ⁺ /bile acid cotransporter protein
2.55	AT5A_03265	Glycerol-3-phosphate dehydrogenase
2.01	AT5A_04120	mRNA 3'-end processing factor
3.43	AT5A_04240	RhtB family transporter
3.77	AT5A_04335	ThiJ/Pfp family protein
2.23	AT5A_05095	MFS permease
3.80	AT5A_06090	CoA-binding domain protein
2.17	AT5A_06770	Glutathione S-transferase
2.00	AT5A_07425	ThyA Thymidylate synthase
2.00	AT5A_07635	LpxC UDP-3-O-acyl-N-acetylglucosamine deacetylase
2.29	AT5A_09480	Glutamine synthetase translation inhibitor
2.59	AT5A_09930	GntR family transcriptional regulator
2.13	AT5A_10095	N-methylproline demethylase
3.45	AT5A_10115	Iron ABC transporter substrate-binding protein
2.03	AT5A_10767	PhnM Ribose 1,5-bisphosphate phosphokinase
2.05	AT5A_13222	Fumarylacetoacetate hydrolase
3.52	AT5A_14197	Ribonucleotide-diphosphate reductase subunit alpha
2.08	AT5A_14562	Dehydrogenase
2.55	AT5A_15197	Acyloxyacyl hydrolase
2.01	AT5A_16516	Mannonate dehydratase
2.25	AT5A_16881	Amino acid ABC transporter permease
2.79	AT5A_17161	Acetyltransferase
2.19	AT5A_17521	HslIU ATP-dependent protease ATPase subunit
2.10	AT5A_18546	PhhB Putative pterin-4-alpha-carbinolamine dehydratase
2.37	AT5A_21536	AmpC beta-lactamase
2.40	AT5A_21641	Zinc-binding dehydrogenase
1.99	AT5A_22256	RraA Ribonuclease activity regulator
2.12	AT5A_22276	UgpE 3n-glycerol-3-phosphate transport system permease
2.27	AT5A_22301	Lipopolysaccharide biosynthesis protein
2.53	AT5A_22851	Aspartate racemase
2.24	AT5A_23935	Beta-Ig-H3/fasciclin
2.00	AT5A_25070	Cytochrome c, class I
19.01	AT5A_25680	Major facilitator transporter
67.74	AT5A_25695	Monooxygenase
12.28	AT5A_25700	Hypothetical protein - universal stress protein related
Genes Activated		
Amino Acid Transport		
-2.13	AT5A_19211	ABC transporter-like protein
-2.48	AT5A_21601	ABC transporter ATPase
-2.23	AT5A_21616	Amino acid ABC transporter substrate-binding protein
-2.06	AT5A_22531	Alpha-ketoglutarate permease

Table 6. Continued.

Genes Activated		
Chemotaxis		
-2.05	AT5A_10165	Chemotaxis protein histidine kinase
-2.05	AT5A_12222	Methyl-accepting chemotaxis protein
-2.48	AT5A_12862	Methyl-accepting chemotaxis protein
-2.44	AT5A_24110	Methyl-accepting chemotaxis protein
Nitrogen Transport		
-2.12	AT5A_03300	NrtC Nitrate transport ATP-binding protein
-2.20	AT5A_03305	NrtB Nitrate transport permease
-2.69	AT5A_03310	NrtA Nitrate transport protein
Sulfate Transport		
-2.06	AT5A_01700	CysA Sulfate/thiosulfate import ATP-binding protein
-2.12	AT5A_01705	Sulfate transport system permease
-2.13	AT5A_05640	Sulfate permease
Queuosine Biosynthesis, Purine Metabolism		
-2.63	AT5A_00855	QueE 7-carboxy-7-deazaguanine synthase
-2.55	AT5A_00860	6-carboxy-5,6,7,8-tetrahydropterin synthase
-2.82	AT5A_00865	QueC 7-cyano-7-deazaguanine synthase
Membrane Protein		
-2.20	AT5A_08260	PA-phosphatase related phosphoesterase
-2.35	AT5A_13767	Membrane protein COG4125
-2.17	AT5A_19206	Polar amino acid ABC transporter inner membrane
Amino Acid Biosynthesis		
-2.08	AT5A_07270	IlvC Ketol-acid reductoisomerase
-2.07	AT5A_02380	D-3-phosphoglycerate dehydrogenase
-2.00	AT5A_19201	ArgH Argininosuccinate lyase
Glycosyl Transfer		
-2.14	AT5A_01910	Glycosyltransferase
-2.26	AT5A_01915	Glycosyltransferase
Signal Transduction		
-2.03	AT5A_09495	Two component sensor kinase
-2.20	AT5A_10180	Metal dependent phosphohydrolase
Miscellaneous		
-2.26	AT5A_02245	Membrane protein
-2.25	AT5A_02655	RpmJ 50S ribosomal protein L36
-2.60	AT5A_05965	Tyrosine/serine protein phosphatase
-2.43	AT5A_07275	TetR family transcriptional regulator
-2.06	AT5A_08270	Lipid A biosynthesis protein
-2.12	AT5A_09225	OccM Octopine transport system permease protein
-2.23	AT5A_14692	Phage head-tail adaptor
-2.16	AT5A_15841	Methionine import ATP-binding protein MetN
-2.20	AT5A_16681	Glycine/betaine ABC transporter substrate-binding
-2.83	AT5A_18146	Nitrogen regulatory protein II
-2.00	AT5A_19221	MmgE/PrpD family protein
-2.52	AT5A_21621	GntR family transcriptional regulator

Genes Regulated by ArsR3 Only. Transcript profile comparisons suggested that loss of ArsR3 resulted in fewest changes; 15 repressed and 8 activated (Table 7). Functions apparently repressed by ArsR3 involve iron uptake, biotin synthesis, sugar transport, chemotaxis, nitrogenase maturation, and monooxygenase activity. Activated genes did not cluster with respect to related functions, but rather represented a miscellaneous collection of activities that are difficult to envision as a concerted cellular reaction.

Table 7. Gene expression significantly influenced by ArsR3 only. Expression ratios were calculated as gene expression levels in the *arsR3* mutant relative to wild type.

Fold Change	Gene name	Gene Annotation
Genes Repressed		
<u>Iron Homeostasis</u>		
2.35	AT5A_05990	Ferritin
7.68	AT5A_08610	TonB-dependent heme receptor A
5.46	AT5A_23490	TonB-dependent siderophore receptor
<u>Biotin Synthesis</u>		
2.02	AT5A_15067	Cysteine desulfurase
2.35	AT5A_22476	BioD ATP-dependent dethiobiotin synthetase
2.15	AT5A_22481	Adenosylmethionine--8-amino-7-oxononanoate transaminase
<u>Miscellaneous</u>		
2.64	AT5A_23500	Monooxygenase, NtaA/SnaA/SoxA family protein
2.49	AT5A_24030	Membrane Protein
2.40	AT5A_24050	Sugar ABC transporter permease
2.29	AT5A_24060	Carbohydrate Binding Protein
2.27	AT5A_11942	Photosystem Reaction Center Subunit H
2.22	AT5A_21661	Methyl-accepting chemotaxis serine transducer
2.17	AT5A_15087	NifS Nitrogenase cofactor synthesis protein
Genes Activated		
<u>Miscellaneous</u>		
-2.35	AT5A_09745	Putative tricarboxylic transport membrane protein
-2.19	AT5A_16111	Protocatechuate 3,4-dioxygenase, beta subunit
-2.19	AT5A_16016	Branched amino acid ABC transporter substrate binding protein
-2.16	AT5A_16151	3-oxoadipate CoA-transferase subunit B
-2.10	AT5A_09755	TctA Tripartite tricarboxylate transporter
-2.07	AT5A_16106	Protocatechuate 3,4-dioxygenase, alpha subunit
-2.07	AT5A_12077	Bacteriophage repressor protein C1

Genes Regulated by ArsR4 Only. Transcriptional patterns indicated ArsR4 significantly influenced more genes than ArsR3, but fewer than ArsR2 and ArsR1. A

total of 60 genes appear influenced, with 37 of these genes being unique to ArsR4 (Table 8). Most (34 in total) apparently are repressed and correspond to three main themes: a Fix operon normally associated with N₂ fixation, universal stress response, and pyruvate metabolism. Of the two genes exhibiting decreased expression, the most interesting is *arsR2* and indicates that ArsR2 levels are influenced by ArsR4, but in an activating fashion instead of as a repressor.

Table 8. Gene expression significantly influenced uniquely by *ArsR4*. Expression ratios were calculated as gene expression levels in the *arsR4* mutant relative to wild type.

Fold Change	Gene name	Gene Annotation
Genes Repressed		
<u>Respiration</u>		
2.68	AT5A_13282	FixI Nitrogen fixation protein
3.41	AT5A_13287	FixH Nitrogen fixation protein
4.44	AT5A_13292	FixG Nitrogen fixation protein
2.80	AT5A_13312	Cbb3-type cytochrome c oxidase subunit
3.01	AT5A_13317	FixQ chain Cytochrome-c oxidase
3.34	AT5A_13322	FixO chain Cytochrome-c oxidase
4.07	AT5A_13327	Cbb3-type cytochrome c oxidase subunit I
4.52	AT5A_19736	Nodulin-related transmembrane protein
2.55	AT5A_22901	Cytochrome D ubiquinol oxidase subunit I
2.05	AT5A_22906	Cytochrome d ubiquinol oxidase, subunit II
<u>Universal Stress Response</u>		
3.32	AT5A_19291	Hypothetical Protein
3.29	AT5A_19296	Hypothetical Protein
3.23	AT5A_19281	UspA Universal Stress Protein
<u>Pyruvate Metabolism</u>		
3.36	AT5A_19266	AckA Acetate kinase
2.71	AT5A_11862	Acetyl-CoA hydrolase
<u>Miscellaneous</u>		
5.15	AT5A_00100	Outer membrane protein
4.26	AT5A_13577	Coproporphyrinogen-III oxidase
4.09	AT5A_09375	Regulator of biofilm formation, Fnr family protein
3.99	AT5A_19306	Pyridoxine oxidase
3.98	AT5A_19731	OsmC family protein
2.95	AT5A_12772	Alcohol dehydrogenase, zinc-containing
2.86	AT5A_16781	HlyD family secretion protein
2.72	AT5A_08590	Pseudoazurin
2.64	AT5A_23261	Hypothetical protein
2.45	AT5A_18861	5-aminolevulinate synthase
2.40	AT5A_04835	Carboxymuconolactone decarboxylase
2.24	AT5A_16786	ABC transporter, nucleotide binding/ATPase protein
2.06	AT5A_26415	Lipid A oxidase
Genes Activated		
<u>Miscellaneous</u>		
-2.79	AT5A_03370	Glycosyl transferase family protein
-1.99	AT5A_25685	ArsR2 family transcriptional regulator

As(III) influences on ArsR regulatory control

It was also of interest to examine how As(III) might influence gene expression profiles in the absence of the different ArsRs. This would provide important information regarding how the regulatory function of these proteins might potentially change as a consequence of binding As(III). For example, significant research (Kang et al., 2016; Rosen, 1999, 2002) supports the current view that ArsR undergoes a conformational change upon binding As(III), causing it to release from its DNA binding site and thus allowing the RNA polymerase access to an otherwise blocked promoter. It is at least conceivable that this same conformational change may now cause these proteins to target other DNA sequences, resulting in As(III)-associated repression or activation. In such a scenario, decreased gene expression when comparing +As(III) mutants against +As(III) wild type would infer that the missing ArsR is directly or indirectly involved in enhancing expression of the gene when bound by As(III). By contrast, increased expression in this comparison would imply that there is some type of As(III)-based activation mechanism at play and able to exhibit its activity in the absence of the ArsR protein; i.e., the As(III) bound ArsR has a repressor-like function. These gene expression profiles would only be recognizable in a mutant. With this as context, Table 9 to Table 12 summarize changes observed for cell function categories in which large numbers of genes appear influenced positively or negatively; i.e., large and consistent transcription response patterns.

Arsenic Resistance. Expression changes of the different components of the arsenic resistance response ranged from modest to very significant (Table 9). These changes were ArsR-dependent and included both increased and decreased expression, inferring that when As(III)-bound the different ArsR proteins continued exerting repressor activity or now assumed an activator function, respectively. Of the eight genes that were further As(III)-upregulated in the mutants, expression of three *arsR* transcriptional repressors, *arsR3*, *arsR5* (AT5A_25245), and an orphan *arsR*, increased by 2 to 42 fold. Elements of the *arsR4* operon (*arsH2*, *arsC3*, *acr3-2*) increased ~2 fold in the $\Delta arsR1$ mutant and is consistent with these genes being controlled by ArsR1 (Table 5).

Table 9. Influence of As(III) on ArsR control of the arsenic resistance response. As(III) levels used were 100 μ M, 50 μ M, 75 μ M, and 75 μ M for the $\Delta arsR1$, $\Delta arsR2$, $\Delta arsR3$, and $\Delta arsR4$ mutants, respectively. Values represent positive or negative fold change when comparing the mutant vs. the wild type and blue spacers demarcate genes located in predicted or known operons.

Genome identifier	Gene Annotation	$\Delta arsR1$ mutant	$\Delta arsR2$ mutant	$\Delta arsR3$ mutant	$\Delta arsR4$ mutant
AT5A_08115	Transcriptional regulator, ArsR family	-2.9			
AT5A_22226	ArsR3 family transcriptional regulator		11.4		
AT5A_22236	ArsH2 Arsenical resistance protein	2.2			
AT5A_22241	ArsC3 Arsenate reductase	2.1			
AT5A_22246	Acr3-2 Arsenite efflux pump protein	2.3			
AT5A_25245*	ArsR family transcriptional regulator		42.0		
AT5A_07815	Transcriptional regulator, ArsR family		2.6		2.3

Chemotaxis. As(III) affects on chemotaxis genes appears to be primarily associated with ArsR2 and ArsR4, with some overlap (Table 10). Based on expression levels in the wild type strain, it would appear that transcription of a complete operon fails to exceed the thresholds established for this study 50-75 μ M As(III) and indeed this cluster of genes is down-regulated at the highest level of As(III) tested, suggesting the

possibility that at the highest As(III) levels the cell perceives the metalloid as a repellent. However, at a lower As(III) level, expression levels in the $\Delta arsR4$ mutant are further depressed relative to the wild type cell, suggesting ArsR4 normally activates (~2 fold) these genes.

Table 10. Influence of As(III) on ArsR control of chemotaxis. As(III) levels used were 100 μ M, 50 μ M, 75 μ M, and 75 μ M for the $\Delta arsR1$, $\Delta arsR2$, $\Delta arsR3$ and $\Delta arsR4$ mutants. Values represent positive or negative fold change when comparing the mutant vs the wild type and blue spacers demarcate genes located in predicted or known operons.

Genome Identifier	Gene Annotation/Function	<i>arsR1</i> Mutant	<i>arsR2</i> Mutant	<i>arsR3</i> Mutant	<i>arsR4</i> Mutant
AT5A_02480	Methyl-accepting chemotaxis protein		-2.3		-2.4
AT5A_10140	CheB Chemotaxis response regulator protein		-2.8		-2.4
AT5A_10145	Chemotaxis protein methyltransferase		*		-2.4
AT5A_10150	CheW Chemotaxis protein		*		-2.2
AT5A_10155	Methyl-accepting chemotaxis protein		*		-2.2
AT5A_10160	CheW Chemotaxis protein		*		-2.2
AT5A_10165	Chemotaxis protein histidine kinase		-2.1		-2.5
AT5A_10170	Chemotaxis receiver protein		-2.0		-2.6
AT5A_12222	Methyl-accepting chemotaxis protein				-2.1
AT5A_12862	Methyl-accepting chemotaxis protein A				-2.2
AT5A_18836	Methyl-accepting chemotaxis protein	-2.3			
AT5A_24110	Methyl-accepting chemotaxis protein				-2.2

Sugar Transporters. Three sugar transporter operons were variably influenced by the addition of As(III) to the mutants. Expression of two unspecified transporters was increased in the $\Delta arsR1$ (25-33 fold) and $\Delta arsR2$ mutants (~2-3 fold) (Table 11). Assuming that both ArsR1 and ArsR2 would be As(III) saturated under the conditions employed, this would suggest that normally both of these operons would be significantly repressed. Conversely, in the $\Delta arsR4$ mutant, transcription of an operon annotated as a maltose transporter was reduced, implying As(III)-bound ArsR4 normally activates its transcription.

Table 11. Influence of As(III) on ArsR control of sugar transporters. As(III) levels used were 100 μ M, 50 μ M, 75 μ M, and 75 μ M for the $\triangle arsR1$, $\triangle arsR2$, $\triangle arsR3$ and $\triangle arsR4$ mutants. Values represent positive or negative fold change when comparing the mutant vs the wild type and blue spacers demarcate genes located in predicted or known operons.

Genome Identifier	Gene Annotation/Function	<i>arsR1</i> Mutant	<i>arsR2</i> Mutant	<i>arsR3</i> Mutant	<i>arsR4</i> Mutant
AT5A_18906	Maltose ABC transporter protein				-2.4
AT5A_18911	Maltose ABC transporter transmembrane				-2.4
AT5A_18916	Maltose ABC transporter substrate-binding				-2.7
AT5A_18921	Maltose/maltodextrin ABC transporter				-2.3
AT5A_19366	Sugar ABC transporter periplasmic protein	25.4			
AT5A_19371	Sugar ABC transporter permease	25.4			
AT5A_19376	Multiple sugar transport permease protein	30.7			
AT5A_19386	Sugar ABC transporter ATP-binding protein	33.6			-2.0
AT5A_19781	Ribose ABC transporter protein		2.0		
AT5A_24025	Sugar ABC transporter substrate-binding		2.4		
AT5A_24035	Sugar ABC transporter nucleotide ATPase		2.6		
AT5A_24045	Sugar ABC transporter permease		2.8		
AT5A_24050	Sugar ABC transporter permease		2.6		

Iron homeostasis. Perhaps one of the most interesting observations concerns the affect of As(III) and ArsR proteins on the expression of genes involved in iron acquisition and regulation (Table 12). Again, as with the functions examined above, there was a mixture of increased and decreased expression depending on the ArsR. Iron transporters, TonB and TonB receptors, ferrichrome transporters, heme biosynthesis, and Fe³⁺ tranporters were all affected. While some genes were expressed at higher levels such as with the $\triangle arsR1$ mutant, most affected genes were expressed at lower levels in the $\triangle arsR4$ mutant. These data indicate that ArsR4 is significantly involved in transcriptional activation in response to the cell being challenged by As(III).

Table 12. Influence of As(III) on ArsR control of iron homeostasis. As(III) levels used were 100 μ M, 50 μ M, 75 μ M, and 75 μ M for the $\triangle$ arsR1, $\triangle$ arsR2, $\triangle$ arsR3 and $\triangle$ arsR4 mutants. Values represent positive or negative fold change when comparing the mutant vs the wild type and blue spacers demarcate genes located in predicted or known operons.

Genome Identifier	Gene Annotation/Function	arsR1 Mutant	arsR2 Mutant	arsR3 Mutant	arsR4 Mutant
ATSA_00040	Periplasmic chelated iron-binding protein	-3.0			
ATSA_00050	Iron ABC transporter ATP-binding protein	-2.4	-2.9		
ATSA_01140	Iron ABC transporter nucleotide binding				-2.5
ATSA_01145	Iron ABC transporter membrane spanning				-2.6
ATSA_01150	Iron ABC transporter substrate binding protein				-4.0
ATSA_01155	Iron ABC transporter substrate binding protein				-4.4
ATSA_01170	Iron ABC transporter nucleotide binding/ATPase	2.4			
ATSA_01175	Iron ABC transporter membrane spanning	2.5			
ATSA_01180	Iron ABC transporter substrate binding protein	2.2			
ATSA_05975	Fe III dicitrate ABC transporter, permease	2.1			
ATSA_09670	Iron-chelator utilization protein				-2.2
ATSA_09710	Heme utilization protein HuvX				-4.1
ATSA_09920	Heme oxygenase				-3.6
ATSA_10100	FbpC Fe(3+) ions import ATP-binding protein	-4.8	10.8		5.1
ATSA_10110	Iron ABC transporter substrate-binding protein	-7.3	10.8		10.6
ATSA_10115	Iron ABC transporter substrate-binding protein	-2.8	2.6		3.3
ATSA_10732	Biopolymer transport protein				-4.7
ATSA_10742	TonB protein				-6.4
ATSA_10942	Iron ABC transporter substrate-binding protein				-2.6
ATSA_11337	Iron-regulated protein				-4.8
ATSA_11917	Iron ABC transporter nucleotide-binding				-3.9
ATSA_11922	Iron ABC transporter substrate-binding protein				-3.7
ATSA_11927	Iron ABC transporter transmembrane protein				
ATSA_16416	FitR Iron transport system regulatory protein				-2.1
ATSA_16421	Ferrichrome ABC transporter				
ATSA_16431	Ferrichrome transport system permease				
ATSA_16436	Ferrichrome ABC transporter				-2.6
ATSA_16631	Fe3+ siderophore ABC transporter permease				-2.3
ATSA_16636	Fe3+ siderophore ABC transporter permease				-2.7
ATSA_19866	FecR protein				-5.4
ATSA_22566	Ferrichrome ABC transporter				-5.3
ATSA_22571	Iron-hydroxamate transporter permease		-3.5		-4.5
ATSA_22576	Ferrichrome ABC transporter				-4.1
ATSA_23490	TonB-dependent siderophore receptor				-2.1
ATSA_23505	Iron ABC transporter, ATP binding protein				-2.5
ATSA_23515	Iron ABC transporter permease				-2.7
ATSA_23520	Iron ABC transporter periplasmic				-3.6
ATSA_23525	Siderophore-interacting protein				-3.0
ATSA_23555	Enterobactin synthase subunit E				-2.5
ATSA_23560	2,3-dihydro-2,3-dihydroxybenzoate synthetase				

Discussion

The regulatory roles of the ArsR proteins and purpose of their reiteration has been studied, but never before on a genome-wide scale. The influence of the ArsR proteins found in this study is greater than ever previously reported. We found the ArsR proteins regulate several different functions in the cell. This study gives further insight into the

purpose of the *arsR* reiteration, the ArsR proteins activator activities, and identifies several functions that are influenced by the ArsR proteins.

Purpose of *arsR* Reiteration

Several reasons for the reiteration of the *arsR* genes have been explored. In one organism, *P. putida* KT2440, maximal efficiency at different temperatures is thought to be the purpose of the *arsR* reiteration. One *ars* operon functions best below a certain temperature, and the other functions best above a certain temperature (Paez-Espino et al., 2015). However, there is little difference in the function of the *ars* operons in *A. tumefaciens* due to temperature differences (Kang et al., 2016). In this bacterium, ArsR1, ArsR2, and ArsR4 respond at different levels of As(III) exposure and it has also been shown to a limited extent that each of the ArsR proteins are involved in distinct processes (Kang et al., 2016).

Multiple *arsR* genes may be needed in *A. tumefaciens* to participate in a ArsR regulatory network and delegate control of specific functions in the cell. A complex regulatory hierarchy exists between the ArsR proteins (Figure 10) in which ArsR1 represses *arsR4*, ArsR4 activates *arsR2*, and ArsR2 exerted repressive regulatory control over *arsR3*. There were genes that were regulated by multiple ArsR proteins. More experimentation with multiple *arsR* knockout mutants is needed to determine the effects of this multiple ArsR regulation. In addition, the ArsR proteins each uniquely regulated

several genes. For example, ArsR1 had a regulatory effect on *phoB1*, but ArsR2, ArsR3, and ArsR4 did not (Table 5). This indicated a distinct role for each of the ArsR proteins.

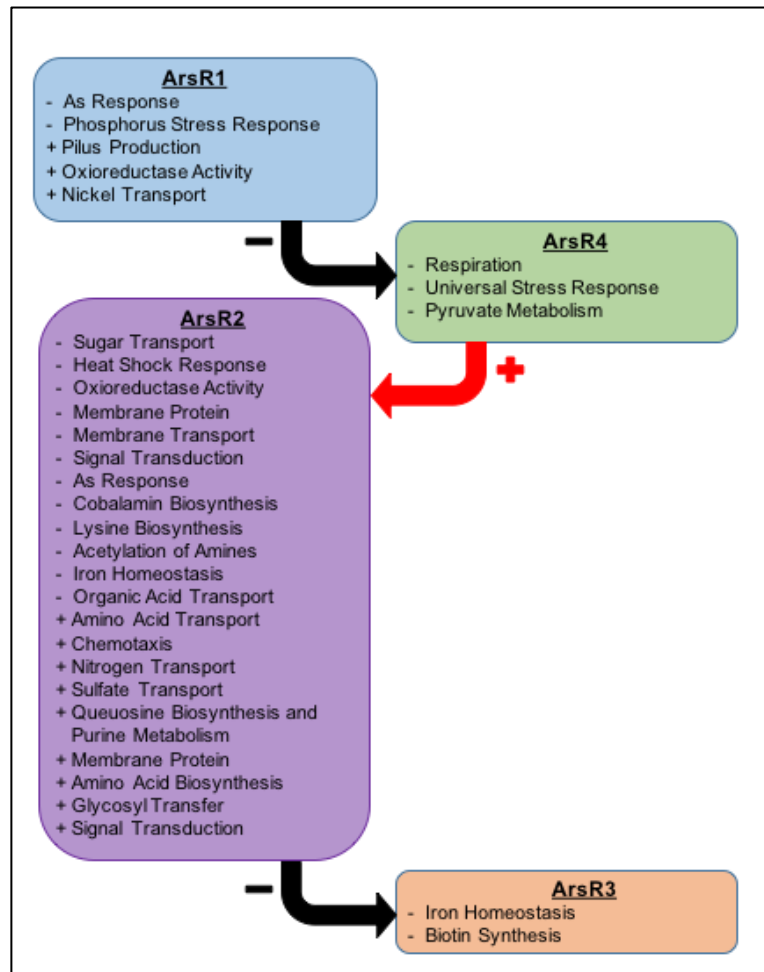


Figure 10. ArsR Regulatory Hierarchy. The ArsR proteins were found to have a regulatory hierarchy with ArsR1 repressing *arsR4*, ArsR4 activating *arsR2*, and ArsR2 repressing *arsR3*. Only functions with at least two genes showing significant expression changes (as determined by comparing gene expression levels in the *arsR* mutant to the wildtype) were included in this figure. The functions that are shown in this figure are exclusive to the ArsR protein; i.e. As response is exclusively regulated by ArsR1 and displayed no changes due to ArsR2, ArsR3, and ArsR4. The '-' symbol indicates repression and the '+' symbol indicates activation.

ArsR1 appeared to play the largest regulatory role in the As response out of any of the ArsR proteins. It repressed the most As response genes and most of these genes were exclusively controlled (not regulated by ArsR2, ArsR3, or ArsR4). This holds with the idea of ArsR1 being at the “top” of the As(III) response regulatory hierarchy. Although ArsR1 did not regulate the most genes overall (ArsR2 influenced the greatest number of genes), it is clearly the main ArsR regulator in the As(III) response.

ArsR3 was previously thought to be non-response to As(III) levels, and based on position and orientation it was proposed that *arsR3* may be regulated with an upstream *lysR* gene (Kang et al., 2016). However, in this study *arsR3* expression was increased when the wild type cells were exposed to 100 μ M As(III). The differential gene expression analysis of RNA sequence in this study was more sensitive than the reverse transcriptase PCR used in the study that showed ArsR3 was non-responsive to As(III) levels, so it was able to detect this change. ArsR3 may be responding directly to the As(III) levels or the lack of repression effects when ArsR2 disassociates with the DNA in response to As(III) levels, which could lead to ArsR3 displaying sensitivity to As(III) in the environment through a cascade effect.

Activator function

The ArsR proteins were originally thought to only have repressive regulatory capabilities. However, a recent study using *arsR* deletion mutants indicated that ArsR proteins may have activator function (Kang et al., 2016). Our study showed multiple activation activities for the ArsR proteins. When comparing the *arsR* deletion mutants to the wild type under the same conditions, expression of 120 genes was down regulated

(Table 3). This indicated these genes were activated by the respective ArsR proteins, meaning over 1/3 of the regulatory activities of the ArsR proteins resulted in gene activation.

Functions Regulated by the ArsR Proteins

All of the ArsR proteins influenced functions outside of their respective *ars* operon (Figure 11). Evidence of ArsR proteins having regulatory influence outside of their operon has been demonstrated in the past. Notably, ArsR1 exerts regulatory control over *phoB1* and *pstS1* genes, which are involved in the cellular phosphorus stress response (Kang, Heinemann, et al., 2012). However, the number of genes and variety of functions affected by the ArsR proteins as shown in this study is unprecedented. ArsR proteins have regulatory influence over several genes involved in various functions in the cell.

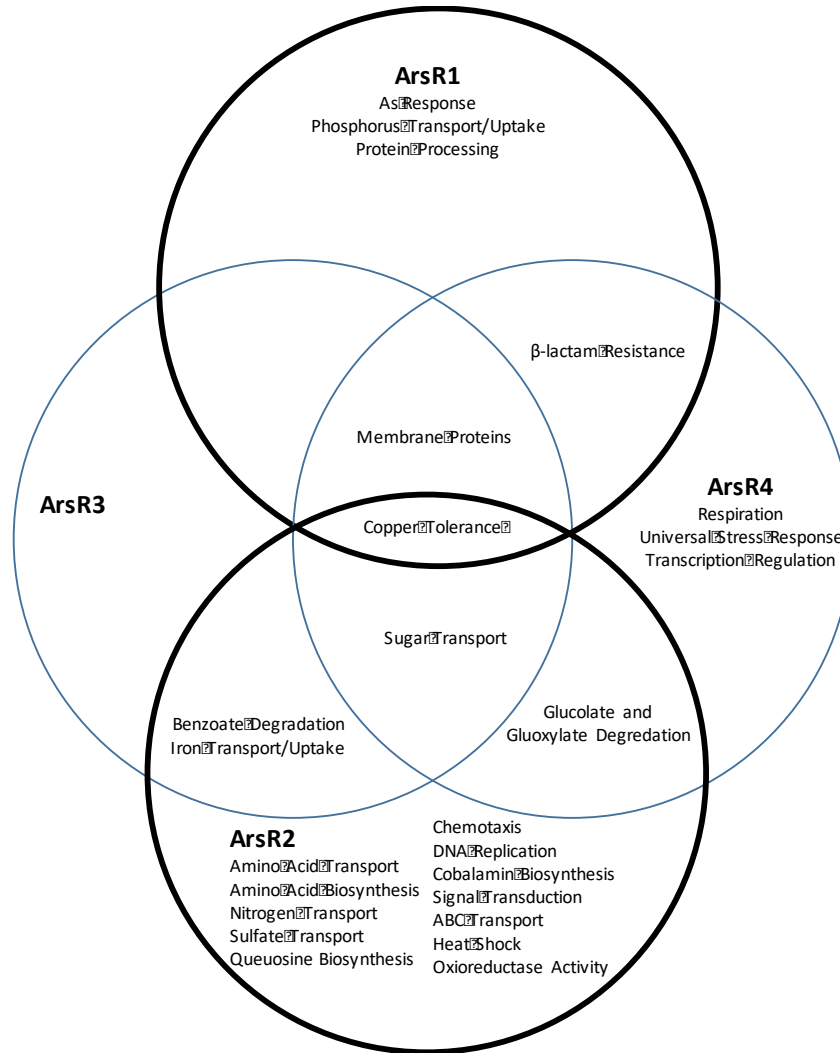


Figure 11. Functions Regulated by the ArsR Proteins. Functions with at least three related genes regulated by the ArsR protein are included in this Venn diagram. Regulation was determined by significant change in the gene's expression level in the *arsR* mutant over the wild type.

The relationship between ArsR1 and changes in bacterial phosphorus acquisition/metabolism has already been established (Kang, Heinemann, et al., 2012). This study was consistent with this relationship finding 11 phosphorus acquisition/metabolism genes were repressed by ArsR1 (Table 5). The other ArsR proteins appeared to have little role in phosphorus metabolism in the cell, as ArsR2

repressed only one phosphonate transporter, and ArsR3 and ArsR4 had no regulatory effect. We saw increased expression levels in 13 phosphorus acquisition/metabolism genes as the As(III) levels were increased in the wild type cell (Table 2).

All four of the ArsR proteins repressed sugar transport genes, with ArsR2 repressing the most (Table 11). Thus, it appears that the ArsR proteins play a key role in sugar transport in the As(III) stressed cell. However, this increase in sugar transporters is not easy to explain. Although previous research has shown that exposure to As(III) affects carbon metabolism in bacteria, it does not necessarily provide a reason for cells to increase the sugar transporters (Tokmina-Lukaszewska et al., 2017).

Expression of several genes related to iron homeostasis was affected by the ArsR proteins. All four of the ArsR proteins regulated at least one gene related to iron homeostasis when comparing expression levels in the *arsR* mutants to the wild type. Ferritin was repressed by ArsR1 and ArsR3 when no As(III) was present. Ferritin not only regulates intracellular iron levels, but may also serve as a cytoprotective protein by sequestering intracellular iron and thus minimizing oxygen free radical formation (Orino et al., 2001). Thus, ArsR proteins may not only affect iron homeostasis, but may also provide protection for the cell from the reactive oxygen species. In addition, multiple iron homeostasis genes showed decreased expression when comparing the *arsR1*, *arsR2* and especially *arsR4* mutants with As(III) exposure to the wild type with As(III) exposure (Table 12). This indicated an activating role for *arsR1*, *arsR2*, and *arsR4* in iron homeostasis during times of As(III) stress.

Copper tolerance genes were activated by all four of the ArsR proteins, with ArsR1 and ArsR4 activating the most. As contaminated environments often contain heavy metals which may indicate a need for multi-resistances. There is a trend of finding copper tolerance and As resistance genes on the same plasmid (Hao et al., 2017). For example, in hydrothermal vents many As resistant bacteria were found to have copper resistance genes in their plasmid (Farias et al., 2015).

Arsenic oxidizing bacteria have been shown to display positive chemotaxis towards As(III). A recent paper explored the regulatory mechanism of this chemotaxis in *A. tumefaciens*. A gene, *mcp*, was found to be responsible for the positive chemotaxis. *mcp* is located in the As island and regulated by AioR (Shi et al., 2017). ArsR2 activated five proteins involved in chemotaxis when As(III) was not present in this study (Table . In addition, ArsR4 activated 11 genes, ArsR1 activated one gene, and ArsR2 activated four chemotaxis genes when in the presence of As(III) (Table 10). These results indicated that the ArsR1, ArsR2, and ArsR4 proteins also play a role in activating chemotaxis.

A gene coding for glutamate dehydrogenase (GDH) was repressed by all four ArsR proteins (Table 4). GDH uses ammonia and α -ketoglutarate to synthesize glutamate. An additional ammonia is incorporated into glutamate by the enzyme glutamine synthetase to form glutamine (Berg 2002). Levels of glutamate and glutamine have been shown to be increased in bacterial cells exposed to As(III) (Tokmina-Lukaszewska et al., 2017). This is due to a key enzyme in the TCA cycle, α -ketoglutarate dehydrogenase, being inhibited by As(III) leaving an excess of α -ketoglutarate in the cell

(Tokmina-Lukaszewska et al., 2017). The ArsR proteins would increase transcription of the gene coding for glutamate dehydrogenase in times of As(III) stress. This could be to compensate for the excess α -ketoglutarate in an As(III) stressed cell. The α -ketoglutarate could be used by GDH to produce the glutamate and glutamine, both of which are upregulated in As(III) stressed cells(Tokmina-Lukaszewska et al., 2017).

The arsenite oxidase large subunit, coded by the *aioA* gene, was repressed by all four ArsR proteins (Table 4) and upregulated by 75 μ M As(III). *aioB*, which codes for the arsenite oxidase small subunit, is found adjacent to *aioA*(Slyemi & Bonnefoy, 2012). These subunits together form an arsenite oxidase complex. Expression of *aioB* was not found to be significantly changed in any of the mutants. This raises questions as to whether there is an independent function for AioA, however no such function has ever been proposed.

The ArsR proteins repressed seven heat shock response and five universal stress response genes (Table 3). Heat shock and universal stress response genes are upregulated in response to stressful conditions encountered by bacteria, including metal/metalloid exposure. Heat shock proteins play important roles in physiological processes such as stress tolerance and protein chaperoning (Mahmood, Jadoon, Mahmood, Irshad, & Hussain, 2014; Taipale, Jarosz, & Lindquist, 2010). Universal stress response proteins increase survival during stressful situations by providing oxidative stress resistance, motility, adhesion, and iron homeostasis(Nachin, Nannmark, & Nystrom, 2005).

Six genes related to β -lactam antibiotic resistance were activated by the ArsR proteins (Table 3). Studies have shown that bacteria with multiple heavy metal

resistances often show resistance to antibiotics that may not even be found in their environment. One example is a study in a high-altitudes Andean wetland, where there is no antibiotic selective pressure (Dib, Motok, Zenoff, Ordonez, & Farias, 2008). This study found a correlation between UV-B radiation, As resistance, and antibiotic resistance (Dib et al., 2008). Another study in Antarctic marine waters showed resistance to both antibiotics and metals/metalloids was common in the bacterial isolates (De Souza, Nair, Bharathi, & Chandramohan, 2006). There appears to be some correlation between As resistance and antibiotic resistance, and the ArsR proteins may play a key role.

Significance

This study demonstrated the regulatory influence of the ArsR proteins on many cellular functions. Several major cycles in the cell were influenced by one or more of the ArsR proteins including: carbon (sugar transport), nitrogen (nitrogen transport), sulfur (sulfate transport), and phosphorus (phosphorus acquisition/metabolism). If we use *A. tumefaciens* as a model for As-microbe interactions, these changes in the bacterial cell's cycling of nutrients may add up to making a significant impact in biogeochemical cycling of these nutrients through an ecosystem. To this end, understanding these changes is crucial. In addition, there are several methods that rely on microbial As speciation change in order to cleanup As contamination in water and soil environments. Understanding microbe interactions with As is important so they can be utilized to their full potential. Thus, understanding the regulatory proteins in a system such as the *ars* operons in *A. tumefaciens* gives further insight into biogeochemical cycling in ecosystems, as well as potential for cleanup of As contaminated sites.

REFERENCES CITED

- Achour-Rokbani, A., Cordi, A., Poupin, P., Bauda, P., & Billard, P. (2010). Characterization of the ars Gene Cluster from Extremely Arsenic-Resistant *Microbacterium* sp Strain A33. *Applied and Environmental Microbiology*, 76(3), 948-955. doi:10.1128/aem.01738-09
- ATSDR. (2011). Arsenic Toxicity Where is Arsenic Found. Retrieved from <http://www.atsdr.cdc.gov/csem/csem.asp?csem=1&po=5>
- ATSDR. (2016). ATSDR – Priority List of Hazardous Substances. Retrieved from <http://www.atsdr.cdc.gov/spl/>
- Branco, R., Chung, A. P., & Morais, P. V. (2008). Sequencing and expression of two arsenic resistance operons with different functions in the highly arsenic-resistant strain *Ochrobactrum tritici* SCII24(T). *Bmc Microbiology*, 8, 12. doi:10.1186/1471-2180-8-95
- Butcher, B. G., & Rawlings, D. E. (2002). The divergent chromosomal ars operon of *Acidithiobacillus ferrooxidans* is regulated by an atypical ArsR protein. *Microbiology-Sgm*, 148, 3983-3992.
- Cai, L., Liu, G. H., Rensing, C., & Wang, G. J. (2009). Genes involved in arsenic transformation and resistance associated with different levels of arsenic-contaminated soils. *Bmc Microbiology*, 9, 11. doi:10.1186/1471-2180-9-4
- Chauhan, N. S., Ranjan, R., Purohit, H. J., Kalia, V. C., & Sharma, R. (2009). Identification of genes conferring arsenic resistance to *Escherichia coli* from an effluent treatment plant sludge metagenomic library. *Fems Microbiology Ecology*, 67(1), 130-139. doi:10.1111/j.1574-6941.2008.00613.x
- Chen, J., Bhattacharjee, H., & Rosen, B. P. (2015). ArsH is an organoarsenical oxidase that confers resistance to trivalent forms of the herbicide monosodium methylarsenate and the poultry growth promoter roxarsone. *Molecular Microbiology*, 96(5), 1042-1052. doi:10.1111/mmi.12988
- Chilvers, D. C., Peterson, P.J. (1987). *Lead, Mercury, Cadmium, and Arsenic in the Environment* (T. C. Hutchinson, Meema, K.M Ed.). Chichester: John Wiley and Sons Ltd.
- Cordi, A., Pagnout, C., Devin, S., Poirel, J., Billard, P., Dollard, M. A., & Bauda, P. (2015). Determination of physiological, taxonomic, and molecular characteristics

- of a cultivable arsenic-resistant bacterial community. *Environmental Science and Pollution Research*, 22(18), 13753-13763. doi:10.1007/s11356-014-3840-5
- De Souza, M. J., Nair, S., Bharathi, P. A. L., & Chandramohan, D. (2006). Metal and antibiotic-resistance in psychrotrophic bacteria from Antarctic Marine waters. *Ecotoxicology*, 15(4), 379-384. doi:10.1007/s10646-006-0068-2
- Dey, S., Dou, D. X., Tisa, L. S., & Rosen, B. P. (1994). INTERACTION OF THE CATALYTIC AND THE MEMBRANE SUBUNITS OF AN OXYANION-TRANSLOCATING ATPASE. *Archives of Biochemistry and Biophysics*, 311(2), 418-424. doi:10.1006/abbi.1994.1256
- Dib, J., Motok, J., Zenoff, V. F., Ordonez, O., & Farias, M. E. (2008). Occurrence of resistance to antibiotics, UV-B, and arsenic in bacteria isolated from extreme environments in high-altitude (Above 4400 m) andean wetlands. *Current Microbiology*, 56(5), 510-517. doi:10.1007/s00284-008-9103-2
- Ellis, P. J., Conrads, T., Hille, R., & Kuhn, P. (2001). Crystal structure of the 100 kDa arsenite oxidase from *Alcaligenes faecalis* in two crystal forms at 1.64 angstrom and 2.03 angstrom. *Structure*, 9(2), 125-132. doi:10.1016/s0969-2126(01)00566-4
- EPA. (2013). *Introduction to In Situ Bioremediation of Groundwater*.
- Farias, P., Santo, C. E., Branco, R., Francisco, R., Santos, S., Hansen, L., . . . Morais, P. V. (2015). Natural Hot Spots for Gain of Multiple Resistances: Arsenic and Antibiotic Resistances in Heterotrophic, Aerobic Bacteria from Marine Hydrothermal Vent Fields. *Applied and Environmental Microbiology*, 81(7), 2534-2543. doi:10.1128/aem.03240-14
- Finkelman, R. B., Belkin, H. E., & Zheng, B. S. (1999). Health impacts of domestic coal use in China. *Proceedings of the National Academy of Sciences of the United States of America*, 96(7), 3427-3431. doi:10.1073/pnas.96.7.3427
- Gadd, G. M. (2010). Metals, minerals and microbes: geomicrobiology and bioremediation. *Microbiology-Sgm*, 156, 609-643. doi:10.1099/mic.0.037143-0
- Gihring, T. M., & Banfield, J. F. (2001). Arsenite oxidation and arsenate respiration by a new *Thermus* isolate. *Fems Microbiology Letters*, 204(2), 335-340. doi:10.1111/j.1574-6968.2001.tb10907.x
- Gress, J., da Silva, E. B., de Oliveira, L. M., Zhao, D., Anderson, G., Heard, D., . . . Ma, L. Q. (2016). Potential arsenic exposures in 25 species of zoo animals living in CCA-wood enclosures. *Science of the Total Environment*, 551, 614-621. doi:10.1016/j.scitotenv.2016.02.065

- Hao, X., Li, X., Pal, C., Hobman, J., Larsson, D. G. J., Saquib, Q., . . . Rensing, C. (2017). Bacterial resistance to arsenic protects against protist killing. *Biometals*, 30(2), 307-311. doi:10.1007/s10534-017-0003-4
- Hood, E. (2012). The Apple Bites Back Claiming Old Orchards for Residential Development. *Environmental Health Perspectives*, 120(5), A470-A476.
- Hsieh, Y. J., & Wanner, B. L. (2010). Global regulation by the seven-component Pi signaling system. *Current Opinion in Microbiology*, 13(2), 198-203. doi:10.1016/j.mib.2010.01.014
- Hughes, M. F. (2002). Arsenic toxicity and potential mechanisms of action. *Toxicology Letters*, 133(1), 1-16. doi:10.1016/s0378-4274(02)00084-x
- Kang, Y. S., Bothner, B., Rensing, C., & McDermott, T. R. (2012). Involvement of RpoN in Regulating Bacterial Arsenite Oxidation. *Applied and Environmental Microbiology*, 78(16), 5638-5645. doi:10.1128/aem.00238-12
- Kang, Y. S., Brame, K., Jetter, J., Bothner, B. B., Wang, G. J., Thiyagarajan, S., & McDermott, T. R. (2016). Regulatory Activities of Four ArsR Proteins in *Agrobacterium tumefaciens* 5A. *Applied and Environmental Microbiology*, 82(12), 3471-3480. doi:10.1128/aem.00262-16
- Kang, Y. S., Heinemann, J., Bothner, B., Rensing, C., & McDermott, T. R. (2012). Integrated co-regulation of bacterial arsenic and phosphorus metabolisms. *Environmental Microbiology*, 14(12), 3097-3109. doi:10.1111/j.1462-2920.2012.02881.x
- Kang, Y. S., Shi, Z. J., Bothner, B., Wang, G. J., & McDermott, T. R. (2015). Involvement of the Acr3 and DctA anti-porters in arsenite oxidation in *Agrobacterium tumefaciens* 5A. *Environmental Microbiology*, 17(6), 1950-1962. doi:10.1111/1462-2920.12468
- Kashyap, D. R., Botero, L. M., Franck, W. L., Hassett, D. J., & McDermott, T. R. (2006). Complex regulation of arsenite oxidation in *Agrobacterium tumefaciens*. *Journal of Bacteriology*, 188(3), 1081-1088. doi:10.1128/jb.188.3.1081-1088.2006
- Kashyap, D. R., Botero, L. M., Lehr, C., Hassett, D. J., & McDermott, T. R. (2006). A Na⁺: H⁺ antiporter and a molybdate transporter are essential for arsenite oxidation in *Agrobacterium tumefaciens*. *Journal of Bacteriology*, 188(4), 1577-1584. doi:10.1128/jb.188.4.1577-1584.2006
- Kaur, S., Kamli, M. R., & Ali, A. (2011). Role of arsenic and its resistance in nature. *Canadian Journal of Microbiology*, 57(10), 769-774. doi:10.1139/w11-062

- Khan, B. I., Solo-Gabriele, H. M., Dubey, B. K., Townsend, T. G., & Cai, Y. (2004). Arsenic speciation of solvent-extracted leachate from new and weathered CCA-treated wood. *Environmental Science & Technology*, 38(17), 4527-4534. doi:10.1021/es049598r
- Koechler, S., Cleiss-Arnold, J., Proux, C., Sismeiro, O., Dillies, M. A., Goulhen-Chollet, F., . . . Bertin, P. N. (2010). Multiple controls affect arsenite oxidase gene expression in *Herminiimonas arsenicoxydans*. *Bmc Microbiology*, 10, 13. doi:10.1186/1471-2180-10-53
- Kruger, M. C., Bertin, P. N., Heipieper, H. J., & Arsene-Ploetze, F. (2013). Bacterial metabolism of environmental arsenic-mechanisms and biotechnological applications. *Applied Microbiology and Biotechnology*, 97(9), 3827-3841. doi:10.1007/s00253-013-4838-5
- Kumagai, Y., & Sumi, D. (2007). Arsenic: Signal transduction, transcription factor, and biotransformation involved in cellular response and toxicity. In *Annual Review of Pharmacology and Toxicology* (Vol. 47, pp. 243-262). Palo Alto: Annual Reviews.
- Lamarche, M. G., Wanner, B. L., Crepin, S., & Harel, J. (2008). The phosphate regulon and bacterial virulence: a regulatory network connecting phosphate homeostasis and pathogenesis. *Fems Microbiology Reviews*, 32(3), 461-473. doi:10.1111/j.1574-6976.2008.00101.x
- Landrum, J. T., Bennett, P. C., Engel, A. S., Alsina, M. A., Pasten, P. A., & Milliken, K. (2009). Partitioning geochemistry of arsenic and antimony, El Tatio Geyser Field, Chile. *Applied Geochemistry*, 24(4), 664-676. doi:10.1016/j.apgeochem.2008.12.024
- Lim, K. T., Shukor, M. Y., & Wasoh, H. (2014). Physical, Chemical, and Biological Methods for the Removal of Arsenic Compounds. *Biomed Research International*, 9. doi:10.1155/2014/503784
- Lin, Y. F., Walmsley, A. R., & Rosen, B. P. (2006). An arsenic metallochaperone for an arsenic detoxification pump. *Proceedings of the National Academy of Sciences of the United States of America*, 103(42), 15617-15622. doi:10.1073/pnas.0603974103
- Link, A. J., Phillips, D., & Church, G. M. (1997). Methods for generating precise deletions and insertions in the genome of wild-type *Escherichia coli*: Application to open reading frame characterization. *Journal of Bacteriology*, 179(20), 6228-6237.

- Liu, G. H., Liu, M. Y., Kim, E. H., Maaty, W. S., Bothner, B., Lei, B. F., . . . McDermott, T. R. (2012). A periplasmic arsenite-binding protein involved in regulating arsenite oxidation. *Environmental Microbiology*, 14(7), 1624-1634. doi:10.1111/j.1462-2920.2011.02672.x
- Lopez-Maury, L., Sanchez-Riego, A. M., Reyes, J. C., & Florencio, F. J. (2009). The Glutathione/Glutaredoxin System Is Essential for Arsenate Reduction in *Synechocystis* sp Strain PCC 6803. *Journal of Bacteriology*, 191(11), 3534-3543. doi:10.1128/jb.01798-08
- Mahmood, K., Jadoon, S., Mahmood, Q., Irshad, M., & Hussain, J. (2014). Synergistic Effects of Toxic Elements on Heat Shock Proteins. *Biomed Research International*, 17. doi:10.1155/2014/564136
- Martin, P., DeMel, S., Shi, J., Gladysheva, T., Gatti, D. L., Rosen, B. P., & Edwards, B. F. P. (2001). Insights into the structure, solvation, and mechanism of ArsC arsenate reductase, a novel arsenic detoxification enzyme. *Structure*, 9(11), 1071-1081. doi:10.1016/s0969-2126(01)00672-4
- McCleskey, R. B., Nordstrom, D. K., Susong, D. D., Ball, J. W., & Taylor, H. E. (2010). Source and fate of inorganic solutes in the Gibbon River, Yellowstone National Park, Wyoming, USA. II. Trace element chemistry. *Journal of Volcanology and Geothermal Research*, 196(3-4), 139-155. doi:10.1016/j.jvolgeores.2010.05.004
- McEwan, A. G., Ridge, J. P., McDevitt, C. A., & Hugenholtz, P. (2002). The DMSO reductase family of microbial molybdenum enzymes; Molecular properties and role in the dissimilatory reduction of toxic elements. *Geomicrobiology Journal*, 19(1), 3-21. doi:10.1080/014904502317246138
- Messens, J., & Silver, S. (2006). Arsenate reduction: Thiol cascade chemistry with convergent evolution. *Journal of Molecular Biology*, 362(1), 1-17. doi:10.1016/j.jmb.2006.07.002
- Mukherjee, A. B., & Bhattacharya, P. (2001). Arsenic in groundwater in the Bengal Delta Plain: Slow poisoning in Bangladesh. *Environmental Reviews*, 9(3), 189-220. doi:10.1139/er-9-3-189
- Nachin, L., Nannmark, U., & Nystrom, T. (2005). Differential roles of the universal stress proteins of *Escherichia coli* in oxidative stress resistance, adhesion, and motility. *Journal of Bacteriology*, 187(18), 6265-6272. doi:10.1128/jb.187.18.6265-6272.2005

- Nimick, D. A., Moore, J. N., Dalby, C. E., & Savka, M. W. (1998). The fate of geothermal arsenic in the Madison and Missouri Rivers, Montana and Wyoming. *Water Resources Research*, 34(11), 3051-3067. doi:10.1029/98wr01704
- Nriagu, J. O., & Pacyna, J. M. (1988). QUANTITATIVE ASSESSMENT OF WORLDWIDE CONTAMINATION OF AIR, WATER AND SOILS BY TRACE-METALS. *Nature*, 333(6169), 134-139. doi:10.1038/333134a0
- Ordonez, E., Letek, M., Valbuena, N., Gil, J. A., & Mateos, L. M. (2005). Analysis of genes involved in arsenic resistance in *Corynebacterium glutamicum* ATCC 13032. *Applied and Environmental Microbiology*, 71(10), 6206-6215. doi:10.1128/aem.71.10.6206-6215.2005
- Oremland, R. S., Saltikov, C. W., Wolfe-Simon, F., & Stolz, J. F. (2009). Arsenic in the Evolution of Earth and Extraterrestrial Ecosystems. *Geomicrobiology Journal*, 26(7), 522-536. doi:10.1080/01490450903102525
- Oremland, R. S., & Stolz, J. F. (2003). The ecology of arsenic. *Science*, 300(5621), 939-944. doi:10.1126/science.1081903
- Orino, K., Lehman, L., Tsuji, Y., Ayaki, H., Torti, S. V., & Torti, F. M. (2001). Ferritin and the response to oxidative stress. *Biochemical Journal*, 357, 241-247. doi:10.1042/0264-6021:3570241
- Paez-Espino, A. D., Durante-Rodriguez, G., & de Lorenzo, V. (2015). Functional coexistence of twin arsenic resistance systems in *Pseudomonas putida* KT2440. *Environmental Microbiology*, 17(1), 229-238. doi:10.1111/1462-2920.12464
- Pascua, C. S., Minato, M., Yokoyama, S., & Sato, T. (2007). Uptake of dissolved arsenic during the retrieval of silica from spent geothermal brine. *Geothermics*, 36(3), 230-242. doi:10.1016/j.geothermics.2007.03.001
- Peryea, F. J. (1998, Aug 1998). *Historical use of lead arsenate insecticides, resulting soil contamination and implications for soil remediation*. Paper presented at the 16th World Congress of Soil Science, Montpellier, France.
- Pillai, J. K., Venkadesh, S., Ajees, A. A., Rosen, B. P., & Bhattacharjee, H. (2014). Mutations in the ArsA ATPase that restore interaction with the ArsD metallochaperone. *Biometals*, 27(6), 1263-1275. doi:10.1007/s10534-014-9788-6
- Qin, J., Rosen, B. P., Zhang, Y., Wang, G. J., Franke, S., & Rensing, C. (2006). Arsenic detoxification and evolution of trimethylarsine gas by a microbial arsenite S-adenosylmethionine methyltransferase. *Proceedings of the National Academy of*

- Sciences of the United States of America*, 103(7), 2075-2080.
doi:10.1073/pnas.0506836103
- Rosen, B. P. (1999). Families of arsenic transporters. *Trends in Microbiology*, 7(5), 207-212. doi:10.1016/s0966-842x(99)01494-8
- Rosen, B. P. (2002). Biochemistry of arsenic detoxification. *Febs Letters*, 529(1), 86-92. doi:10.1016/s0014-5793(02)03186-1
- Rosen, B. P., Ajees, A. A., & McDermott, T. R. (2011). Life and death with arsenic
Arsenic life: An analysis of the recent report "A bacterium that can grow by using arsenic instead of phosphorus". *Bioessays*, 33(5), 350-357.
doi:10.1002/bies.201100012
- Santini, J. M., Sly, L. I., Schnagl, R. D., & Macy, J. M. (2000). A new chemolithoautotrophic arsenite-oxidizing bacterium isolated from a gold mine: Phylogenetic, physiological, and preliminary biochemical studies. *Applied and Environmental Microbiology*, 66(1), 92-97.
- Sardiwal, S., Santini, J. M., Osborne, T. H., & Djordjevic, S. (2010). Characterization of a two-component signal transduction system that controls arsenite oxidation in the chemolithoautotroph NT-26. *Fems Microbiology Letters*, 313(1), 20-28. doi:10.1111/j.1574-6968.2010.02121.x
- Schlesinger, W. a. B., Emily. (2013). *Biogeochemistry an analysis of global change* (3rd ed.). Oxford, UK: Elvsevier.
- Schooley, T. (2008). The History of Lead Arsenate Use in Apple Production: Comparison of its Impact in Virginia with Other States. *Journal of Presticide Safety Education*, 10, 22-48.
- Shen, Z. Q., Luangtongkum, T., Qiang, Z. Y., Jeon, B., Wang, L. P., & Zhang, Q. J. (2014). Identification of a Novel Membrane Transporter Mediating Resistance to Organic Arsenic in *Campylobacter jejuni*. *Antimicrobial Agents and Chemotherapy*, 58(4), 2021-2029. doi:10.1128/aac.02137-13
- Shi, K. X., Fan, X., Qiao, Z. X., Han, Y. S., McDermott, T. R., Wang, Q., & Wang, G. J. (2017). Arsenite oxidation regulator AioR regulates bacterial chemotaxis towards arsenite in *Agrobacterium tumefaciens* GW4. *Scientific Reports*, 7, 11. doi:10.1038/srep43252
- Slyemi, D., & Bonnefoy, V. (2012). How prokaryotes deal with arsenic. *Environmental Microbiology Reports*, 4(6), 571-586. doi:10.1111/j.1758-2229.2011.00300.x

- Smedley, P. L., & Kinniburgh, D. G. (2002). A review of the source, behaviour and distribution of arsenic in natural waters. *Applied Geochemistry*, 17(5), 517-568. doi:10.1016/s0883-2927(02)00018-5
- Smith, A. H., Lingas, E. O., & Rahman, M. (2000). Contamination of drinking-water by arsenic in Bangladesh: a public health emergency. *Bulletin of the World Health Organization*, 78(9), 1093-1103.
- Somerville, J. E., & Kahn, M. L. (1983). CLONING OF THE GLUTAMINE SYNTHETASE-I GENE FROM RHIZOBIUM-MELILOTI. *Journal of Bacteriology*, 156(1), 168-176.
- Sprocati, A. R., Alisi, C., Tasso, F., Marconi, P., Sciallo, A., Pinto, V., . . . Cremisini, C. (2012). Effectiveness of a microbial formula, as a bioaugmentation agent, tailored for bioremediation of diesel oil and heavy metal co-contaminated soil. *Process Biochemistry*, 47(11), 1649-1655. doi:10.1016/j.procbio.2011.10.001
- Stilwell, D. (2015). CAES: Arsenic in Pressure Treated Wood. Retrieved from <http://www.ct.gov/caes/cwp/view.asp?a=2824&q=378050>
- Stolz, J. E., Basu, P., Santini, J. M., & Oremland, R. S. (2006). Arsenic and selenium in microbial metabolism. In *Annual Review of Microbiology* (Vol. 60, pp. 107-130). Palo Alto: Annual Reviews.
- Stolz, J. F., & Oremland, R. S. (1999). Bacterial respiration of arsenic and selenium. *Fems Microbiology Reviews*, 23(5), 615-627. doi:10.1111/j.1574-6976.1999.tb00416.x
- Summers, M. L., Denton, M. C., & McDermott, T. R. (1999). Genes coding for phosphotransacetylase and acetate kinase in *Sinorhizobium meliloti* are in an operon that is inducible by phosphate stress and controlled by PhoB. *Journal of Bacteriology*, 181(7), 2217-2224.
- Taipale, M., Jarosz, D. F., & Lindquist, S. (2010). HSP90 at the hub of protein homeostasis: emerging mechanistic insights. *Nature Reviews Molecular Cell Biology*, 11(7), 515-528. doi:10.1038/nrm2918
- Tchounwou, P. (2003). Carcinogenic and Systemic Health Effects Associated with Arsenic. *Toxicological Pathology*, 575-588.
- Tokmina-Lukaszewska, M., Shi, Z. J., Tripet, B., McDermott, T. R., Copie, V., Bothner, B., & Wang, G. J. (2017). Metabolic response of *Agrobacterium tumefaciens* 5A to arsenite. *Environmental Microbiology*, 19(2), 710-721. doi:10.1111/1462-2920.13615

- Toxicological Profile for Arsenic*. (2007). Retrieved from <http://www.atsdr.cdc.gov/toxprofiles/tp2.pdf>.
- Ure, A. M., & Berrow, M. L. (1982). THE ELEMENTAL CONSTITUENTS OF SOILS. In Bowen, H. J. M. (pp. P94-204).
- van Lis, R., Nitschke, W., Duval, S., & Schoepp-Cothenet, B. (2013). Arsenics as bioenergetic substrates. *Biochimica Et Biophysica Acta-Bioenergetics*, 1827(2), 176-188. doi:10.1016/j.bbambio2012.08.007
- Wang, L. R., Chen, S., Xiao, X., Huang, X., You, D. L., Zhou, X. F., & Deng, Z. X. (2006). arsRBOCT arsenic resistance system encoded by linear plasmid pHZ227 in *Streptomyces* sp strain FR-008. *Applied and Environmental Microbiology*, 72(5), 3738-3742. doi:10.1128/aem.72.5.3738-3742.2006
- Webster, J. G., & Nordstrom, D. K. (2003). Geothermal Arsenic. In A. H. Welch & K. G. Stollenwerk (Eds.), *Arsenic in Ground Water: Geochemistry and Occurrence* (pp. 101-125). Boston, MA: Springer US.
- WHO. (2001). *Environmental Health Criteria 224: Arsenic compounds*. Geneva.
- WHO. (2012). Arsenic. Retrieved from <http://www.who.int/mediacentre/factsheets/fs372/en/>
- WHO. (2016). Ten chemicals of major public health concern. Retrieved from http://www.who.int/ipcs/assessment/public_health/chemicals_phc/en/
- Williams, M. (2001). Arsenic in mine waters: an international study. *Environmental Geology*, 40(3), 267-278.
- Ye, J., Yang, H. C., Rosen, B. P., & Bhattacharjee, H. (2007). Crystal structure of the flavoprotein ArsH from *Sinorhizobium meliloti*. *Febs Letters*, 581(21), 3996-4000. doi:10.1016/j.febslet.2007.07.039
- Yoshinaga, M., & Rosen, B. P. (2014). A C center dot As lyase for degradation of environmental organoarsenical herbicides and animal husbandry growth promoters. *Proceedings of the National Academy of Sciences of the United States of America*, 111(21), 7701-7706. doi:10.1073/pnas.1403057111
- Zargar, K., Conrad, A., Bernick, D. L., Lowe, T. M., Stolc, V., Hoeft, S., . . . Saltikov, C. W. (2012). ArxA, a new clade of arsenite oxidase within the DMSO reductase family of molybdenum oxidoreductases. *Environmental Microbiology*, 14(7), 1635-1645. doi:10.1111/j.1462-2920.2012.02722.x

- Zargar, K., Hoefft, S., Oremland, R., & Saltikov, C. W. (2010). Identification of a Novel Arsenite Oxidase Gene, *arxA*, in the Haloalkaliphilic, Arsenite-Oxidizing Bacterium *Alkalilimnicola ehrlichii* Strain MLHE-1. *Journal of Bacteriology*, 192(14), 3755-3762. doi:10.1128/jb.00244-10
- Zhu, Y. G., Yoshinaga, M., Zhao, F. J., & Rosen, B. P. (2014). Earth Abides Arsenic Biotransformations. In R. Jeanloz (Ed.), *Annual Review of Earth and Planetary Sciences*, Vol 42 (Vol. 42, pp. 443-+). Palo Alto: Annual Reviews.