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Diverse Community of Arsenic Resistant Bacteria Display Arsenate Reducing Capabilities

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Abstract

Naturally found in soil and water environments arsenic is toxic to many organisms, carcinogenic to humans, and poses a significant public health risk. Yet communities of bacteria found thriving in arsenic ridden environments have evolved mechanisms to tolerate and exploit both oxidation states of this heavy metal (arsenite and arsenate). As the site of an old arsenic mine, Black Mountain Open Space Park in San Diego, California has yielded concentrations of arsenic in the soil between 111-14,800 ppm. Exceeding average arsenic soil concentrations and levels considered safe in the environment by the World Health Organization, we sought to characterize the phylogenetic diversity and mechanisms of arsenic resistance within the bacterial community living in the soils of the old mine site. 28 unique species of bacteria were isolated and characterized through a minimum inhibitory concentration experiment that assessed the level of bacterial arsenic resistance and a silver nitrate assay that examined the ability for the bacterial species to transform arsenic between its two naturally occurring oxidation states. Based on the results of these experiments, we found a highly diverse community of bacteria with eight species that also had arsenate reducing capabilities.

Introduction

Bacteria are related to some of the oldest forms of life and evolved to represent some of the most diverse living organisms on Earth. Due to their diversity and highly specific mechanisms, humans have harnessed these properties and exploited bacteria for our own benefits. Some examples found in daily life include the use of the antibiotic streptomycin to treat bacterial infections (Sethi et al. 2013), the natural pesticide *Bacillus thuringiensis*, otherwise known and BT (Schnepf et al. 1998) used in agricultural practices, and lactic acid producing bacteria to produce fermented foods such as beers, sourdough, and yogurt (Leroy and De Vuyst 2004). In addition, bacteria's diversity allows them to survive in every habitat including some of the most unhospitable environments.

One extreme environment bacteria have been found surviving and thriving in are soils and waters, highly contaminated, with the toxic metalloid arsenic. Extensive research has primarily focused on studying communities of bacteria residing in the contaminated soil and water environments in India, China, and Bangladesh (Bachate et al. 2009) because of the proximity to human environments. However, no studies have considered the impacts of arsenic contamination on a community of soil dwelling bacteria at Black Mountain Open Space Park in San Diego County.

Located within one of counties with the highest populations in the United States, Black Mountain Open Space Park is the site of an old arsenic mine. Soils surrounding the mine have been found to contain a massive 111-14,800 ppm arsenic which is strikingly higher than average natural levels in the soil containing about 10ppm arsenic (Dunivan et al. 2018). Even the lower concentration threshold found at the Black Mountain Open Space Park is higher than previous

research environments and 11,100 times higher than the World Health Organization's approved level of 10ppb (0.01ppm) arsenic in the environment (World Health Organization). Due to the proximity to neighboring communities the high levels of contamination are especially concerning and a threat to public health because of the toxicity to living organisms.

In understanding the associated implications of this heavy metal on health, the mechanisms of arsenic toxicity to the cell must be considered. As a heavy metal, arsenic is most frequently found in two oxidation states in the environment (specifically physiological pH); both are oxyanions (arsenate (AsO_4^{3-}) and arsenite ($As(OH)_3$)) (Achour et al. 2006, Silver and Phung 2005). The pentavalent form, arsenate (As(V)), can be reduced to the more toxic, trivalent form arsenite, (As(III)), and arsenite can be oxidized back to arsenate (Dunivan et al. 2018). Both oxidation states of arsenic have structural similarities to important biomolecules arsenic that allow them to disrupt essential cellular processes and thus pose a critical threat to cell viability.

Arsenite is structurally similar to glycerol (Silver and Phung 2005) and will enter the cell through aquaglyceroporins embedded in the cell membrane (Yang et al 2012). Once inside the cell, arsenite interacts with the functional thiol groups on proteins that disrupts disulfide bridges and denatures proteins, thus impairing their function (Anchour et al. 2006). Arsenate is structurally similar to inorganic phosphates (Anchour et al. 2006) and is taken into the cell via the phosphate transport systems Pst and Pit (Yang et al. 2012). After entering the cell, arsenate acts as a competitive inhibitor of many phosphate dependent enzymes (Yang et al. 2012), and metabolic reactions that require phosphorylation are disrupted (Achour et al. 2006). Arsenite and arsenate have been collectively found to inhibit up to 200 human enzymes (Ratnaike 2003), yet this disruption is not isolated to multicellular organisms as many single cellular organisms including bacteria, experience the same inhibition of normal enzyme function.

However, due to the selective pressure of arsenic's vast toxicity to a diverse range of enzymes in living organisms, bacteria have evolved mechanisms to combat the toxicity. First, some bacteria exploit arsenic in metabolic processes (Silver and Phung 2005). Arsenite acts as an electron donor for chemolithotrophic species, and arsenate can be used in place of oxygen as a final electron acceptor in anaerobic conditions (Silver and Phung 2005). In addition, bacteria have evolved strategies to transform arsenic into less toxic species via methylation or oxidation (Achour et al. 2006) and employ an arsenite efflux system to detoxify the internal cellular environment (Rosen 2002).

Many arsenic resistance genes have been identified, but perhaps the most widespread, throughout a taxonomically diverse range of bacteria, are the genes encoding the arsenic efflux systems (Achour et al. 2006). Located on an operon, that can be found within the chromosome or on a plasmid, some studies have found that some bacteria have inherited arsenic resistance through vertical gene transfer and other species have acquired arsenic resistance through horizontal gene transfer (Dunivan et al. 2018).

The five gene *arsRDABC* operon involved in the efflux systems has been well studied. This operon is controlled via positive repression (Yang et al. 2012) of a single promoter region. *arsR* encodes for a basal repressor, with arsenite binding sites (Yang et al. 2012) that is essential to the regulation of the arsenic resistance operon (Anchour et al. 2006). *arsD* encodes for a metallochaperone protein that transports arsenite within the cell to the ArsB efflux pump (Yang et al. 2012). *arsA* encodes for an ATPase subunit (Anchour et al. 2006) that enhances the efficiency of the ArsB efflux pump to transport arsenite out of the cell (Rosen 2002). *arsB* encodes for the efflux pump which is an integral membrane protein used to transport arsenite out of the cell (Anchour et al. 2006). In some *ars* operons, *arsB* is replaced by an

unrelated *acr3* gene that encodes for a BART (bile/arsenite/riboflavin transporter) superfamily permease (Yang et al. 2012). Finally, *arsC* encodes for an arsenate reductase (Rosen 2002) is responsible for the reduction of arsenate to arsenite, that can be expelled from the cell.

Although arsenate and arsenite can enter the cell by exploiting transport systems designed for other essential biomolecules, the efflux pump critical for internal cell detoxification can only transport arsenite. Arsenite will flow down the glycerol concentration gradient into the cell and can either flow out of the cell passively through ArsB or actively pumped out of the cell by the ArsAB complex. Whereas arsenate must, first, be reduced by ArsC before it can be transported out of the cell. It was proposed that Earth's early, reduced environment allowed for arensite to predominant over arsenate, thus the first detoxification systems only evolved mechanisms to remove arsenite from the cell (Yang et al. 2012). Then as the environment shifted to become more oxidizing and levels of arsenate rose, many bacteria acquired an arsenate reductase gene rather than evolving a separate arsenate efflux system (Yang et al. 2012).

Despite the extensive knowledge surrounding arsenic resistant bacteria, many studies have focused on contaminated soil and water environments abroad and few have considered areas of arsenic contamination within the United States. Therefore, the Bird research lab sought to investigate the soil dwelling bacteria surrounding an old arsenic mine in Black Mountain Open Space Park San Diego, CA. Soil samples collected outside the Black Mountain mine were found to contain 111-14,800 ppm arsenic; even the lower concentration threshold is strikingly higher than most contaminated environments and are 11,100 times higher than the World Health Organization's approved level of 10ppb (0.01ppm) (World Health Organization). By studying the bacterial community residing in this novel environment, insight to the survival mechanisms exploited by these organisms are a first step in developing bioremediation techniques to detoxify

the arsenic soil contamination. Building upon previous research conducted in the Bird lab, this study sought to characterize the arsenite and arsenate minimum inhibitory concentrations of isolated species and assess and their arsenic transformation capacities through a colorimetric silver nitrate assay.

Materials & Methods

28 species of bacteria were isolated from arsenic contaminated soils collected from Black Mountain Open Space Park in San Diego, California by previous researchers in the Bird Lab. Arsenic concentrations in the soil samples ranged from 111ppm to 14,800ppm. All bacteria were grown on R2A plates or in R2A broth medium and were incubated at 30 °C. Two experiments were conducted to characterize the arsenic resistant properties.

A minimum inhibitory concentration (MIC) experiment was conducted to determine the concentrations of arsenate and arsenite that inhibits the growth of each species. Each species was plated in triplicate on R2A plates containing increasing concentrations of arsenite and arsenate utilizing a grid plate method (Supplemental Figure 1). The concentrations of arsenite, in the medium, were: 3.5, 7, 14, 28, 56, and 112 mM and the concentrations of arsenate were: 10, 20, 40, 80, 160, and 360 mM. Plates were incubated for 48 hours before the concentration at which their growth was inhibited (their MIC) was recorded.

A modified silver nitrate assay adopted from Simeonova et al. 2004 and Dunivan et al. 2018 was used to assess the arsenite oxidizing and arsenate reducing capacity of the isolated species. Isolates were inoculated into 8mL R2A broth and incubated for on 72 hours on a rotary shaker (200rpm). After the incubation period, cultures were diluted or concentrated to an optical density of 0.4-0.6 (λ=600nm) with R2A broth. 1mL of the adjusted cultures were pelleted down

in a centrifuge at 8000 rpm for 3 minutes before being washed with 1mL of double distilled water (ddH_2O). Cells were washed an additional 2 times before being suspended in 1.2 ddH_2O. 20µL of the washed cells were incubated with 80µL of 0.2 Tris H-Cl Buffer solution containing arsenate or arsenite in a 96-well plate. Species were plated in triplicate for the arsenate and arsenite conditions and then were incubated for 72 hours at 30°C. Cell suspensions were then checked for viability by utilizing the grid plate method on an R2A plate. Standard curves with varying concentrations of arsenate and arsenite were added to the 96-well plate (0:100, 10:90, 25:75, 50:50, 75:25, 90:10, 100:0). Reaction was initiated by adding 100µL of 0.1M $AgNO_3$ then colorimetric changes were recorded.

Results

Prior work done by lab members Elena Beattle, Lexi Morgan, Stephanie Katz, and Lauren Rubidoux isolated soil species from the Black Mountain mine, performed microscopy, utilized polymerase chain reaction (PCR) and DNA sequencing to identify species based on the 16S rRNA gene and to assess isolates for the *arsC* gene (Supplemental Table 1). Based on the 16S rRNA data a phylogenetic tree was constructed to visualize the evolutionary relationships of the species (Figure 1).

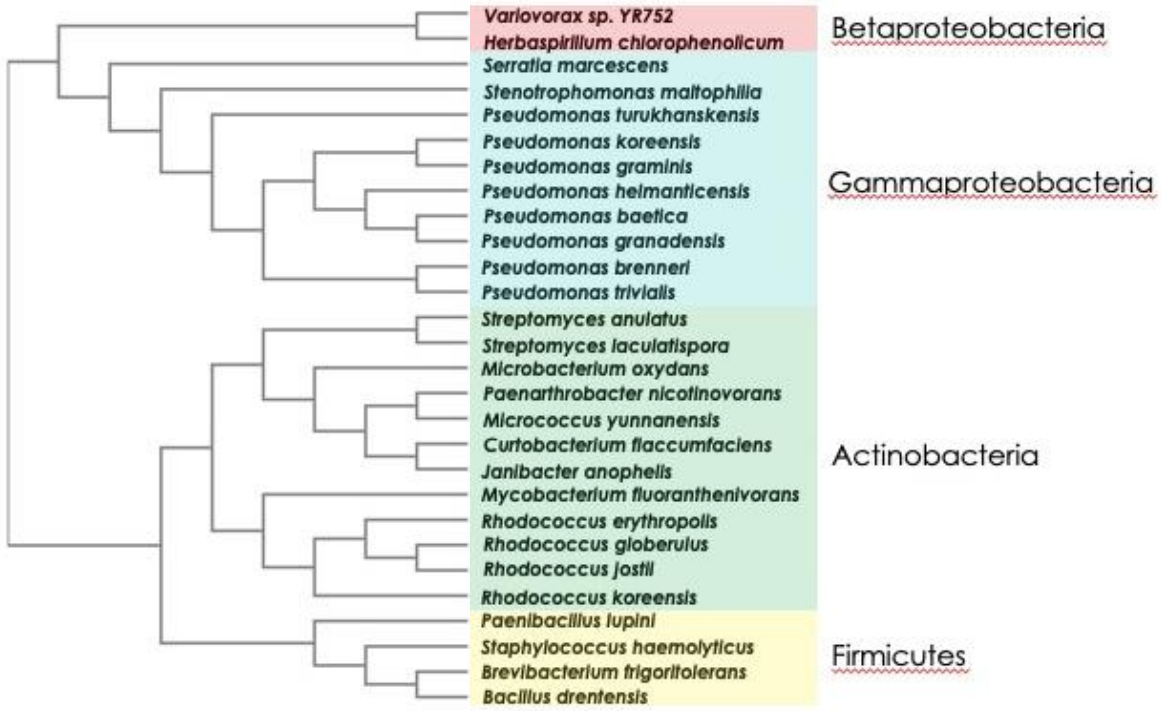

Figure 1 Phylogenetic tree of 16S rRNA sequences from Black Mountain mine isolates.

All species were capable of growing on R2A plates containing arsenite and arsenate and displayed a range of Minimum Inhibitory Concentrations (MICs). MICs for arsenite ranged from 3.5mM to >112mM (Figure 2). 21 of the 28 species had a MIC greater than or equal to 14mM of arsenite. Two species displayed ~~a~~ particularly high MICs to arsenite; *Microbacterium oxydans*

had an MIC of 112mM arsenite and *Rhodococcus jostii* had an MIC >112mM. Both isolates are members of the phylum Actinobacteria.

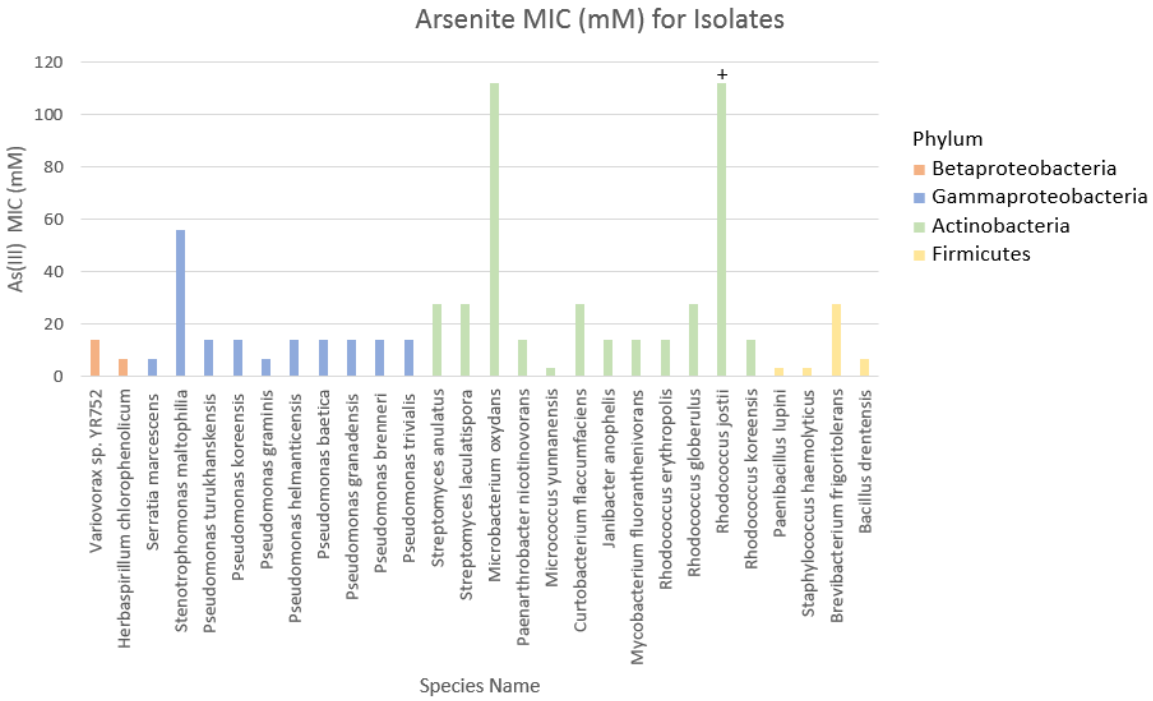

Figure 2 Minimum inhibitory concentration for arsenite (mM) for all isolates.

MICs for arsenate ranged from 20mM to >360mM (Figure 3). Only three of the 28 species had an MIC for arsenate less than 160mM; those three species were *Paenibacillus lupini*, *Staphylococcus haemolyticus*, and *Bascillus drentensis.* These three species were gram positive and from the phylum Firmicutes. 18 of the 28 species had an arsenate MIC >320mM and represented both gram negative and gram positive bacteria. Again, both *Microbacterium oxydans* and *Rhodococcus jostii* displayed high MICs (both >320mM of arsenate). Supplemental Table 1 displays each species' arsenite and arsente MIC in columns three and four, respectively.

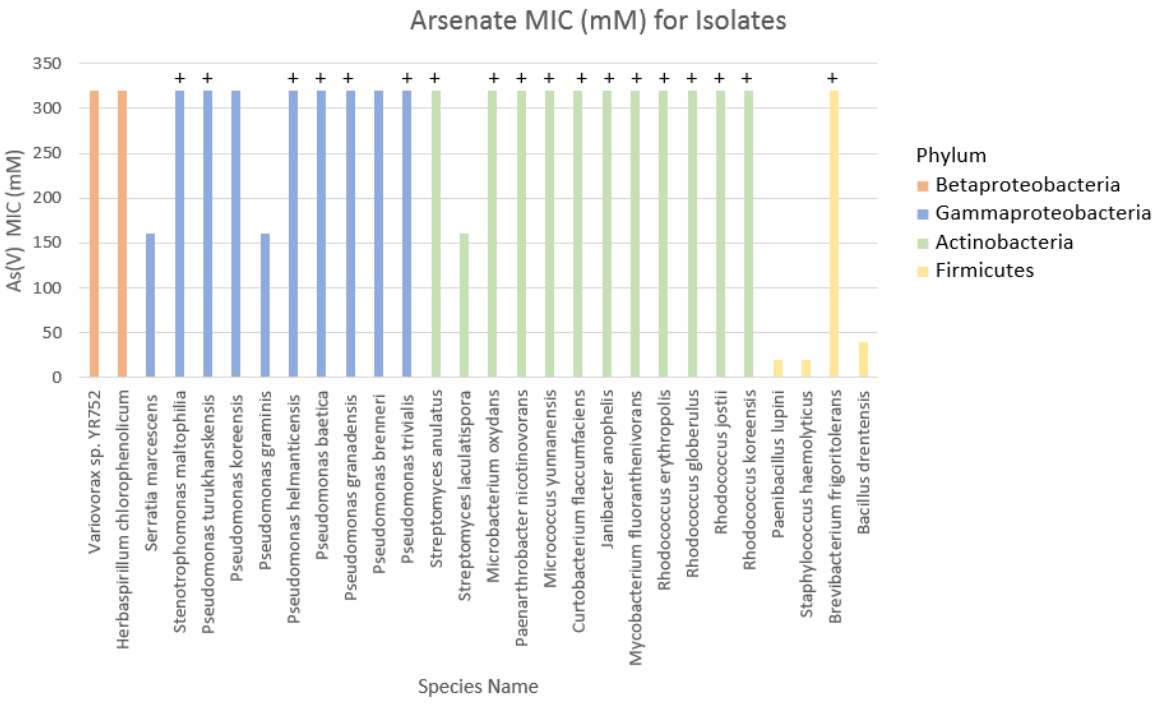

Figure 3 Minimum inhibitory concentration for arsenate (mM) for all isolates.

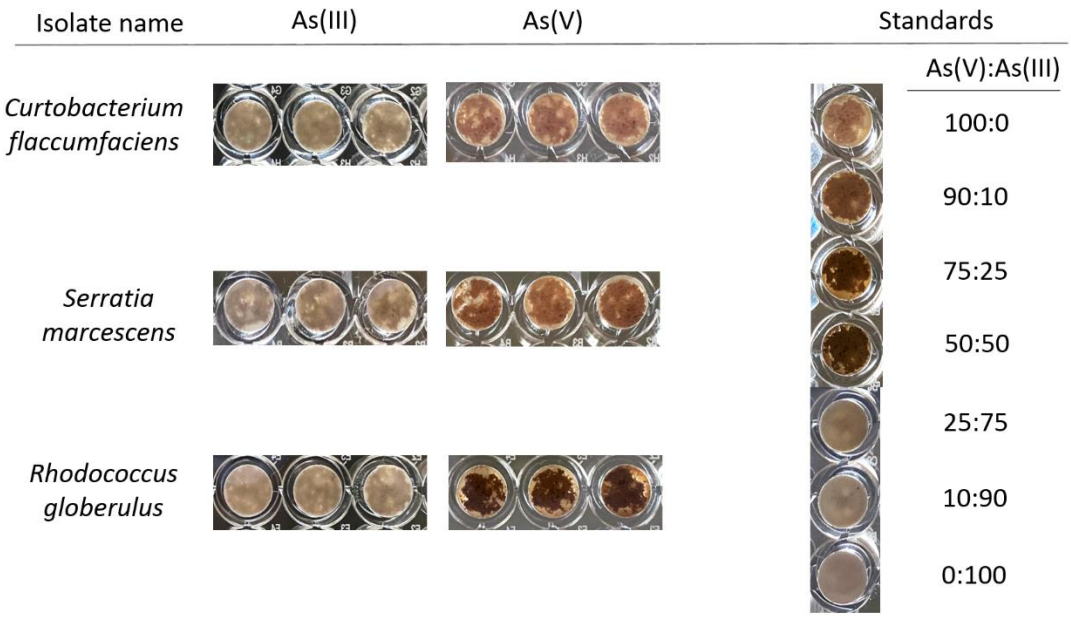

Figure 4 Select results of isolates with a range of arsenate reducing capacities from silver nitrate colorimetric assay.

Figure 4 displays three isolates that had varying arsenate reducing capacities and an example of the arsenic standard curve that were used as a comparison for redox capacities. None of the three species demonstrated arsenite oxidizing capability. *Curtobacterium flaccumfaciens* did not exhibit arsenate reducing activity. *Serratia marcescens* exhibited slight arsenate reducing activity, and *Rhodococcus globerulus* exhibited moderate arsenate reducing activity. Overall results from the colorimetric silver nitrate assay did not detect any species that were able to oxidize arsenite (Supplemental table 1). Eight species displayed the ability to reduce arsenate (Supplemental table 1 and Table 1). Isolates represented members that were both gram negative and gram positive with relatively high arsenate MICs and a mix of arsenite MICs. Six of the eight species that demonstrated arsenate reducing capability possess *arsC,* and two of the species did not have the arsenate reductase gene (*Pseudomonas helmanticensis* and *Rhodococcus globerulus)*.

Table 1 Eight representatives of gram negative and gram positive isolates displayed arsenate reducing capabilities

Species Name	Gram -/+	MIC As(III) (mM)	MIC As(V) (mM)	As(III) oxidizing	As(V) reducing	*arsC*
Serratia marcescens	-	7	160	-	+	+
Pseudomonas helmanticensis	-	14	>320	-	+	+
Pseudomonas granadensis	-	14	>320	-	+	-
Janibacter anophelis	+	14	>320	-	+	+

Rhodococcus erythropolis	+	14	>320	-	+	+
Rhodococcus globerulus	+	28	>320	-	+	+
Rhodococcus jostii	+	>112	>320	-	+	-
Brevibacterium frigoritolerans	+	28	>320	-	+	+

Discussion

Previous work in the Bird research lab isolated and identified 28 unique species from the soils surrounding the former arsenic mine in Black Mountain Open Space Park. 12 species were gram negative and 16 species were gram positive representing a diverse bacterial community. The minimum inhibitory concentration experiment yielded two species with high arsenite MICs (*Microbacterium oxydans* and *Rhodococcus jostii*) and 22 species with high arsenate MICs. Despite the arsenate being tested at higher concentrations, more bacterial species had higher arsenate MICs. The results are consistent with arsenite being the more toxic than arsenate (Dunivan et al. 2018).

It has been proposed that arsenite's toxicity is largely due to the differences in how the uptake systems function (Jomova et al. 2011). Arsenite enters cells through GlpF aquaglyceroporin (Yang et al. 2012, Kalia and Joshi 2009) via passive transport. In high osmolarity conditions the GlpF aquaglyceroporin will close and prevent both glycerol and arsenite uptake (Kalia and Joshi 2009). In contrast, arsenate uptake is much less common because of the selectivity of inorganic phosphate transporters. There are two bacterial phosphate transport systems. The Pit phosphate transport system is constitutively expressed. As a permease

transport channel (Kalia and Joshi 2009), inorganic phosphate and arsenate are transported via passive diffusion down a concentration gradient. Yet, intracellular concentrations of inorganic phosphates remain high when phosphotransferase substrates, like glucose, are present (Rosenberg et al. 1982) so diffusion of arsenate and inorganic phosphates only occur in low phosphotransferase, high arsenate, and high inorganic phosphate environments. As a low affinity, high capacity phosphate intake system (Yang et al. 2012), Pit cannot distinguish between arsenate and inorganic phosphates because arsenate has a K_i equivalent to the K_m of inorganic phosphate (25 μM) (Kalia and Joshi 2009). The Pst phosphate transport system is induced in environments where inorganic phosphate is in low concentrations and there are low intracellular concentrations of inorganic phosphates (Hudek et al. 2016). In contrast to the Pit system, the high selectivity of the Pst transport system (K_m 0.25pM), allows for the distinction of arsenate and prevent its uptake by the Pst system (Kalia and Joshi 2009). Due to the different kinetics of these uptake systems, this can explain why arsenite is the more toxic oxidation state and the observed lower arsenite minimum inhibitory concentrations.

The arsenic transformation assay sought to determine if any of the 28 bacterial isolates were able to convert arsenic as a mechanism of resistance by either using the heavy metal in the electron transport chain or were reducing arsenate with the arsenic reductase, ArsC. Overall, none of the 28 isolates were found to oxidize arsenite; these results are consistent with the findings of Dunivan et al. This suggests that none of the isolates were able to use arsenite as an electron donor in the electron transport chain and were not chemolithotrophic. This result can also be explained by the *arsRDABC* operon system. Within this system of arsenic resistance none of the genes encode for a protein that has a function of oxidizing arsenite because the detoxification system is already adapted to transport arsenite out of the cell. This hypothesis is

based on arsenite being the predominate oxidation state in Earth's early environment. As the Earth become more oxidizing, much of the arsenite was converted to arsenate (Yang et al. 2012). Coinciding with the oxidation event, this system of arsenic resistance evolved the *arsC* instead of evolving a completely new arsenate efflux and detoxification system.

Of the eight bacterial isolates that demonstrated arsenate reduction, six had the *arsC* gene suggesting that these bacterial species can reduce arsenate with ArsC or utilize arsenate as a final electron acceptor in the electron transport chain, in place of oxygen. These species also represented gram negative and gram positive species suggesting that *arsC* is either an ancient gene that is highly conserved or acquired by these species through horizontal gene transfer. In contrast the two species that did not have *arsC* but displayed arsenate reducing capabilities may only be able to reduce arsenate by using it as a final electron acceptor in anaerobic respiration.

There are several limitations of the two experiments that may have affected the results of our study. First, the minimum inhibitory concentration experiment was reliable as our results were consistent with experiments previously done in the Bird research lab. However, the MIC experiment does not provide the exact minimum inhibitory concentrations of arsenite and arsenate to which the isolates are tolerant. Testing a lower starting concentration of arsenite would have provided greater insight as to the exact arsenite MICs and testing higher concentrations of arsenate would have provided greater insight as to the exact arsenate MICs. Secondly, the silver nitrate assay is highly sensitive and only produces qualitative results. By rerunning the silver nitrate assay with higher volumes of arsenic buffer and liquid culture may enhance the visibility of the color change.

Finally, the future directions of this research project would be to scan the remaining species for the presence of the *arsC* gene then building a phylogenetic tree of our bacterial

species based on the evolutionary relationship of *arsC.* This tree would then be compared to the 16S rRNA phylogenetic tree to assess if *arsC* was acquired from horizontal or vertical transmission within our species. Overall, we would then be able to understand the evolutionary relationship of arsenic resistance within the soil dwelling bacterial community of the Black Mountain former arsenic mine site.

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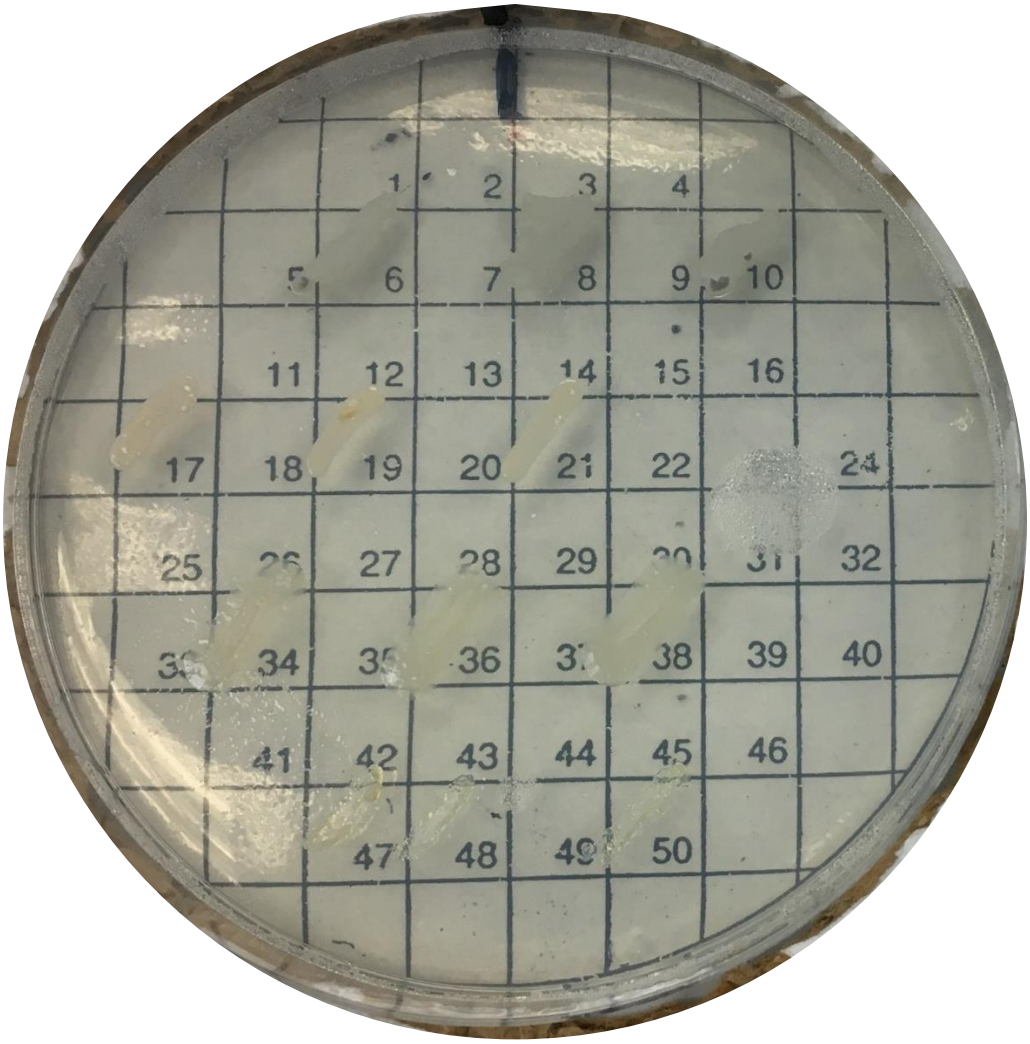
Supplemental Figures



Supplemental Figure 1. Example of R2A grid plate used in the minimum inhibitory concentration experiment. Grid plate has a concentration of 28 mM arsenite and demonstrates four species plated in triplicate.

ST 1 Table summarizing the characteristics of the 28 species. Columns 3 and 4 present experimental MICs and columns 5 and 6 detail results of the silver nitrate assay

Species Name	Gram -/+	MIC As(III) (mM)	MIC As(V) (mM)	As(III) oxidizing	As(V) reducing	*arsC*
Variovorax sp. YR752	-	14	320	-	-	+
Herbaspirillum chlorophenolicum	-	7	320	-	-	-
Serratia marcescens	-	7	160	-	+	+
Stenotrophomonas maltophilia	-	56	>320	-	-	N/A
Pseudomonas turukhanskensis	-	14	>320	-	-	-
Pseudomonas koreensis	-	14	320	-	-	-
Pseudomonas graminis	-	7	160	-	-	+
Pseudomonas helmanticensis	-	14	>320	-	+	+
Pseudomonas baetica	-	14	>320	-	-	-
Pseudomonas granadensis	-	14	>320	-	+	-
Pseudomonas brenneri	-	14	320	-	-	N/A
Pseudomonas trivialis	-	14	>320	-	-	N/A
Streptomyces anulatus	+	28	>320	-	-	+
Streptomyces laculatispora	+	28	160	-	-	+

Microbacterium oxydans	+	112	>320	-	-	+
Paenarthrobacter nicotinovorans	+	14	>320	-	-	+
Micrococcus yunnanensis	+	3.5	>320	-	-	+
Curtobacterium flaccumfaciens	+	28	>320	-	-	+
Janibacter anophelis	+	14	>320	-	+	+
Mycobacterium fluoranthenivorans	+	14	>320	-	-	-
Rhodococcus erythropolis	+	14	>320	-	+	+
Rhodococcus globerulus	+	28	>320	-	+	+
Rhodococcus jostii	+	>112	>320	-	+	-
Rhodococcus koreensis	+	14	>320	-	-	+
Paenibacillus lupini	+	3.5	20	-	-	+
Staphylococcus haemolyticus	+	3.5	20	-	-	+
Brevibacterium frigoritolerans	+	28	>320	-	+	+
Bacillus drentensis	+	7	40	-	-	+