Isolation of Arsenite-Oxidizing Bacteria with Plant Growth Promoting Characteristics to Enhance Phytoremediation of Arsenic

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Paridhi Singh

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Abstract

A common strategy for remediation of arsenic is bacterial remediation by oxidation of As(III) to the less toxic As(V). Another widely accepted technique for arsenic remediation is phytoremediation however in highly contaminated soil, growth of the plants is stunted. Phytoremediation of arsenic is often assisted with plant growth promoting bacteria to improve plant growth. However, there are only a handful of studies on bacteria that can oxidize arsenite as well as promoting plant growth, much less on the implications that they can improve the growth of phytoremediating plants in arsenic contaminated soil. In this study, a group of plant growth promoting arsenite-oxidizing bacteria (PGP As(III)-oxidizing bacteria) from the root zone of plants established in the tailing of the Premier Gold Mine, Beardmore, Ontario was isolated and characterized and their ability to promote the growth of *Trifolium pratense* (red clover) in arsenite contaminated condition was investigated. Thirty-four arsenic resistant bacteria were isolated from the soil collected at the tailing site that was highly contaminated with arsenic (1208 ± 582 ug/g). The isolates were tested both qualitatively and quantitatively for their arsenite-oxidizing ability by using the silver nitrate test and the APDC/ICP-AES analysis, respectively, and all of them demonstrated the ability to oxidize arsenite. The isolates were identified by 16S rDNA sequencing, and a phylogenetic tree was constructed using the maximum likelihood method. The 34 isolates were highly diverse including six different genera. The isolates were tested for four different plant growth promoting characteristics: (i) phosphate solubilization, (ii) siderophore production, (iii) IAA (indole-3-acetic acid) production and (iv) presence of ACC (1-aminocyclopropane-1-carboxylic acid) deaminase. All the isolates showed one or more traits that were tested. Out of the 34 isolates, BS2 and BS21 were selected for further studies based on their diversity, growth rate, arsenite-oxidation ability and presence of different plant growth promoting traits for further sequencing. BS2 and BS21 were identified as *Pseudomonas* sp. and *Rhodococcus* sp., respectively.

Their minimum inhibitory concentrations (MICs) were determined as well as their optimal growth temperature. The MIC for BS2 were 21 mM for As(III) and 740 mM for As(V) whereas for BS21, the MICs were 17 mM for As(III) and 570 mM for As(V). The optimal temperature for both BS2 and BS21 was between 25 to 30°C. In addition, BS2 was positive to all the four plant growth promoting characteristics while BS21 was only positive to siderophore and ACC deaminase production. To determine the beneficial effect of the PGP As(III)-oxidizing bacteria on *T. pratense* in arsenite contaminated conditions, the germination and yield of *T. pratense* in 0, 0.4, 0.6, 1.0 and 1.5 mM sodium arsenite were compared with and without BS2 or BS21 as inoculum. Without the bacterial inoculum, only 24% of the seeds was germinated at 0.4 mM arsenite and no germination was observed at ≥0.6 mM. Despite the 24% germination at 0.4 mM arsenite, the seedlings died at the end of the experiment after 30 d of growth. Therefore, no plant yield was recorded at any arsenite treatments without bacterial inoculum. BS2 increased the germination rate to 100% at 0.4 and 0.6 mM, and 52% at 1.0 mM arsenite. It also improved the plant growth in 0.4, 0.6 and 1.0 mM arsenite with yield of 3.64, 2.78 and 1.86 mg/plant, respectively. For BS21, the inoculum increased the germination rate to 57 and 18% in 0.4 and 0.6 mM arsenite, respectively. Despite the improvement in germination, none of the seedlings inoculated by BS21 in the arsenite treatments grew beyond the seed leaf stage and most of the seedlings eventually died at the end of the experiment. This study shows the potential of PGP As(III)-oxidizing bacteria for improving plant growth in highly arsenic contaminated condition which is important to tackle the problem of arsenic pollution in the environment.

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1. Literature Review

1.1. Introduction

Arsenic is a ubiquitous element found naturally in the Earth's crust and is the 20th most abundant element (NRC, 1977). Some of the sources associated with its natural presence are volcanic rocks, more specifically, their weathering products and ash; hydrothermal ore deposits and their associated geothermal water bodies, marine sedimentary rocks, and fossil fuels (Korte and Fernando, 1991; Smedley and Kinniburgh, 2002). Several natural phenomena, such as weathering of rocks, volcanic activity, and biological activity, in combination with anthropogenic inputs like smelting, fossil fuel processing and combustion, wood preserving, production and applications of pesticides among many others are responsible for contributing to the release or emission of arsenic into the environment (Popovic et al., 2001; Bhattacharya et al., 2002; Wang and Mulligan, 2006). The average concentration of arsenic in the earth's crust is about 2-5 ppm (USDHHS, 2000). Since both natural and anthropogenic factors are variable according to the geography of the area, the arsenic concentrations also vary substantially. These values can diverge from as low as 0.3 ppm, like in certain regions of Southern Manitoba to several thousand ppm (Haluschak et al., 1998). However, as previously mentioned, human activities like mining and smelting can drastically affect the arsenic concentration of an area. For example, in Yellowknife, Northwest Territories, Canada, the tailings of mines contained up to 25,000 ppm arsenic (Ollson, 1999).

Chemically, arsenic is a metalloid that exists in four different oxidation states -3 like in arsine gas (AsH_3), 0 for elemental arsenic, +3 for arsenites and +5 for arsenates. Of these, arsenites and arsenates are the most prevalent in nature (Harper and Haswell, 1988; Savage et al, 2000; Valberg et al., 1997). While arsenite is common in anaerobic conditions, arsenate is mostly found in aerobic environments (DesChamps et al., 2003; Goh and Lim, 2005). Arsenic also occurs as

organometallic species such as monomethylarsonous acid [MMA(III)], monomethylarsonic acid [MMA(V)], dimethylarsinous acid [DMA(III)], dimethylarsinic [DMA(V)], ureidophenylarsonic acid, Arsenobetaine and arsenocholine. Some organic arsenic compounds have been extensively used in agriculture as pesticides and herbicides like dimethylarsinic acid in cotton fields which has resulted in elevated arsenic concentrations in groundwater and soil in those fields as well as nearby water bodies (Bednar et al., 2002). Organoarsenics are found in organisms that have been exposed to arsenic. For instance, different arsenic compounds have been found in marine organisms like the presence of arsenobetaine in crustaceans and arsenosugars in seaweeds. Due to the low toxicity of organoarsenates, the accumulation of these compounds is a lot higher than their inorganic counterparts in living organisms (Lam et al., 2004). The widespread presence of arsenic has led to organisms evolving mechanisms to deal with its toxicity (Leist et al., 2000). Many bacterial species have developed resistance to arsenic toxicity. One of the most important detoxification mechanism is their ability to oxidize As(III) to As(V), which can be 60X less toxic than the As(III) (Abbas et al., 2018). The conversion of As(III) by bacteria is used for remediation purposes and is called bacterial bioremediation.

A variety of plants have also been found to decrease arsenic contamination or toxicity in soil by phytoremediation. Phytoremediation can be achieved by different mechanisms, such as stabilizing or decreasing the mobility of arsenic (Phytostabilization) or extracting arsenic into above ground biomass which can be removed (Phytoextraction). Phytoremediation is a widely used and accepted method for cleanup because it's low cost and can be done in-situ (Salt et al., 1995). However, high concentration of arsenic can decrease or inhibit the growth of the plants and reduce the success of phytoremediation. To increase the growth of plants in arsenic contaminated soil, arsenic-resistant bacteria that show an ability to promote plant growth have been used in combination with plants

for remediation purposes (Ghosh et al., 2011; Wang et al., 2011; Glick, 2003). Although these plant growth promoting arsenic resistant bacteria can improve the growth of plants, they are not capable of reducing the arsenic toxicity in the soil, especially when arsenite is the prominent species. Arsenite oxidizing bacteria, that are capable of detoxifying arsenic by oxidizing As(III) to As(V), have been well-studied in the last few decades (Abbas et al., 2018), however, their ability to produce plant growth promoting factors and enhance the growth of plants for arsenic phytoremediation has rarely been studied. Considering arsenic contamination is a major challenge for the reclamation of mining areas, plant growth promoting As(III)-oxidizing bacteria (PGP As(III)-oxidizing bacteria) could substantially enhance effectiveness of As phytoremediation.

1.2. Arsenic Toxicity

As previously mentioned, arsenic is an element that can have toxic effects on organisms. The mobility and toxicity of the different arsenic species differ based on their chemical forms and their oxidation states (NRC, 1999; Thomas et al., 2001). Inorganic forms are considered to be more toxic than organoarsenate species and amongst the inorganic forms, arsenite is more toxic and mobile than arsenates in groundwater (Gulens et al., 1978). The trivalent form of arsenic has been found to be 60 times more toxic than the pentavalent form (Abbas et al., 2018). Arsenate toxicity happens due to its similar structure to phosphate and its resulting substitution in phosphorylation reactions; however, the products are not very stable and so, they dissociate rapidly. Thus, arsenate is less toxic than arsenite, which can hinder significant chemical processes by reacting with thiols and sulfhydryl groups of molecules like cysteine in proteins (Rosen and Liu 2009).

Inorganic arsenate is about 70 times more toxic than methylated arsenate species like MMA(V) or DMA(V). Other organoarsenate species like arsenosugars, arsenobetaine and arsenocholine are not considered toxic at all (Akter et al., 2005). Methylated arsenite species (MMA(III) and

DMA(III)) are more toxic than the methylated arsenate compounds (Styblo et al., 2000; Dopp et al., 2004), however, studies conducted in natural environments, like freshwater and coastal seawater, show that the concentration of methylated arsenite compounds was about ten to hundred times lower than that of their arsenate counterparts owing to the shorter half life of the methylated arsenite species in the aerobic conditions (Hasegawa, 1996; Hasegawa, 1997; Sohrin et al., 1997). So, methylated arsenite species, be it naturally or due to anthropological factors are scarce in aerobic environments.

Exposure of cells; be it plant, animal or human, to different arsenic species has also been proven to induce production of reactive oxygen species like hydroxyl radical, superoxide etc. (Shi et al., 2004; Hartley-Whitaker et al., 2001; Requejo and Tena, 2005; Singh et al., 2006; Ahsan et al., 2008; Mallick et al., 2011). Mitochondrial membrane potential and its integrity is affected by arsenic and these alterations are believed to be the sites for formation of superoxide anion radicals. These are very harmful and can lead to damage in proteins, nucleic acids, amino acids among many others. They can even cause peroxidation of membrane lipids, which has been observed in *P. vittata* (Møller et al., 2007; Srivastava et al., 2005; Singh et al., 2006).

1.2.1. Toxicity of Arsenate

Arsenate is a compound that has similar structure and properties as phosphate and hence can replace it in a lot of biochemical reactions (Dixon, 1996). Any reaction containing a phosphate or phosphate-ester is a potential target. These can be those essential for cellular metabolism, biosynthesis, information storage and retrieval and cellular signaling, like glycolysis, and oxidative phosphorylation (Orsi and Cleland, 1972; Long and Ray, 1973; Gresser, 1981).

Arsenolysis is a mechanism that can impact the formation of ATP and can happen both at substrate level and mitochondrial level by arsenate replacing phosphate in their respective processes. In the

substrate level, it occurs during the glycolytic pathway. Arsenate replaces phosphate when it's supposed to link enzymatically to D-glyceraldehyde-3-phosphate to form 1,3-biphospho-D-glycerate, instead forming anhydride 1-arsenato-3-phospho-D-glycerate. This anhydride is unstable and hydrolyzes to arsenate and 3- phosphoglycerate. Therefore, ATP is not generated in the presence of arsenate (Crane and Lipmann, 1953; Aposhian, 1989). At the mitochondrial level, this can happen in oxidative phosphorylation where instead of ATP (ADP-Phosphate), in the presence of arsenate and succinate, ADP-Arsenate is synthesized which in turn decreases the ATP production in a cell (Gresser, 1981). Hence at both substrate and mitochondrial levels, this process reduces the formation of ATP.

While most of these studies were done on non-plant systems, it behaves in similar ways in plants and their respective pathways (Finnegan and Chen, 2012). The resemblance in structure of arsenate and phosphate also means that it competes with phosphate for phosphate transporters like the PHT1 proteins in plant systems. Such competition has been shown in many different plant systems like *Arabidopsis thaliana* and *Oryza sativa* (Ullrich-Eberius et al., 1989; Meharg and Macnair, 1992; Clark et al., 2000; Abedin et al., 2002b; Bleeker et al., 2003; Esteban et al., 2003; Tu and Ma, 2003).

1.2.2. Toxicity of Arsenite

Trivalent arsenic compounds can react directly with thiol-containing molecules such as glutathione (GHS) and cysteine (Scott et al., 1993; Delnomdedieu et al., 1994). Since cystine and GSH are essential for most protein molecules, binding of arsenite with any thiol containing enzymes will inactivate their functions by altering their protein folding structures (Cline et al., 2003; Ramadan et al., 2007). Therefore, As(III) is many times more toxic than As(V). Various proteins, among different systems, that are known to be impacted by arsenite include signal

transduction proteins, transcription factors, metabolic and proteolytic enzymes, and structural proteins. Pyruvate dehydrogenase (PDH) is a complex essential for the citric acid cycle as it catalyzes the oxidation of pyruvate to acetyl-CoA. PDH requires lipoic acid, a cofactor for its enzymatic function, but arsenite binds to the lipoic acid disrupting its function (Peters, 1955; Szinicz and Forth, 1988; Hu et al., 1998). Arsenite also targets dihydrolipoamide, which is an essential cofactor of mitochondrial and plastid pyruvate dehydrogenase complexes in plants (Peters et al., 1946; Bergquist et al., 2009).

1.2.3. Toxicity of Organic Arsenics

Monomethylarsonous acid (MMA(III)) and other methylated arsenites are inhibitors of glutathione reductase (Styblo et al., 1997) and thioredoxin reductase (Lin et al., 1999) among other enzymes. Inhibition of such enzymes can lead to cytotoxicity by lowering the efficiency of cellular redox reactions (Hughes, 2002). Since methylated arsenites bind to proteins to a greater extent than their pentavalent counterparts, therefore, they are more toxic to methylated arsenates (Styblo et al., 1995).

1.3. Effects of Arsenic on Human Health

Numerous studies have been conducted in regions with high arsenic concentration in water and soil to determine the correlation between some diseases and exposure to arsenic. People have been exposed to arsenic through ingestion, inhalation, or skin contact. For example, it can accumulate in staple crops like rice; because of irrigation with arsenic contaminated groundwater or application of arsenic containing pesticides like lead arsenate and copper arsenate. For instance, the high concentrations of arsenic found in paddy fields and ground water in the West Bengal (India) and Bangladesh region had inadvertently exposed a large population to arsenic contaminations (Abedin, et al., 2002a).

Studies have found the association between arsenic exposure and cardiovascular diseases (Navas-Acien et al., 2005), neurological disorders (Vahidnia et al., 2007) and even different types of cancer (Miller et al., 2002). Chronic exposure due to sources like drinking water leading to cancer in internal organs like bladder, liver and kidney (Smith et al., 1992; Bates et al., 1995). Skin cancer was also observed in farmers in Taiwan upon drinking water contaminated with arsenic (Tseng, 1968). Long term exposure can lead to cardiovascular problems like cardiac arrhythmias and altered myocardial depolarization (Mumford et al., 2007).

1.4. Arsenic Sources

There are many reasons, be it natural or anthropological, that affect the arsenic concentration in the soil of any region. Some of these have been previously mentioned in the introduction. These sources can be point sources or non-point sources. Widespread application of wood preservatives and pesticides account for most of the non-point source pollutions. Preservatives like chromated copper arsenate (CCA) leads to pollution with the wood distribution that it is applied on. Some pesticides contain arsenic components such as lead arsenate, calcium arsenate, zinc arsenate and/or magnesium arsenate. These have been widely used in agricultural fields in the beginning and mid 20th century. These chemicals seep into the soil and have the potential to pollute ground water and nearby water bodies. The polluted soil and water bodies in the Bangladesh and West Bengal region, as previously mentioned, is a relevant example with some areas containing more than 1000 µg/L in the ground water much higher than the World Health Organization's guideline value of 10 µg/L (Chowdhury et al.,2000).

Point sources for arsenic pollution are usually linked to mining and smelting activities (Sracek et al., 2010). Effluents of smelter as well as base metal refinery, and thermal and power generating stations can increase arsenic concentrations in soil and nearby water bodies. For example, soil

arsenic concentrations near a copper-zinc smelter based in Manitoba, Canada were reported to be 280 ppm, while the average background concentration was just 4 ppm (Zoltai, 1988). Mining activities are one of the anthropological sources with highest arsenic contamination of soil and groundwater of surrounding areas. Mine tailings or effluents hold very high concentrations of arsenic and are one of the most concerning sources of contamination in the environment. For instance, the arsenic levels reached 2200 mg/kg in a lead-zinc mine at Bathurst, New Brunswick, Canada (Wang and Mulligan, 2004), and 4800 and 25000 mg/kg in the Giant Mine tailings and Con Mine tailings in Yellowknife, Northwest Territories, Canada, respectively (Ollson, 1999). Moira Lake in Ontario is one of the areas that had been impacted by arsenic contamination with an annual input of about 3.5 tonnes arsenic from local mining activities (Azcue and Nriagu, 1995).

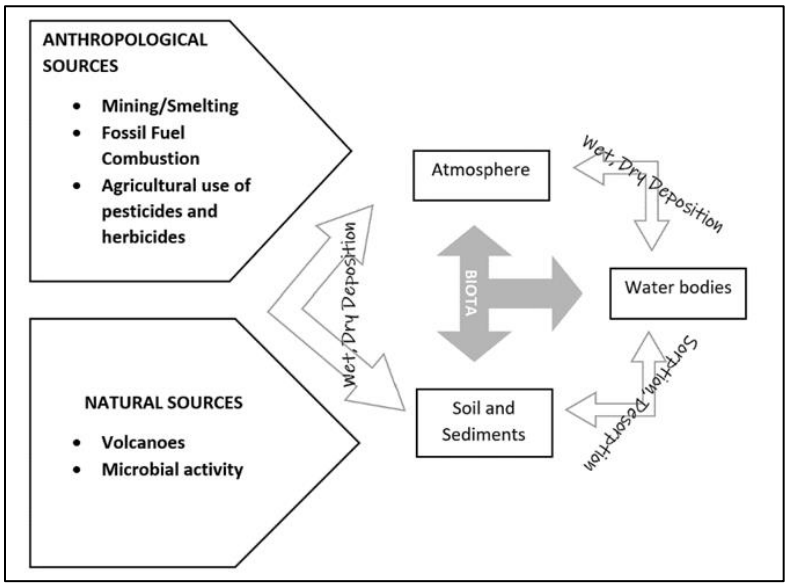

Figure 1.1. Simplified diagram of the arsenic cycle.

1.5. Remediation Techniques

1.5.1. Chemical and Physical Methods for Arsenic Remediation

There are a variety of different methods to deal with the problem of arsenic contamination in soil. Sometimes, the soil is taken to a different site for the remediation process while other times, it can

be done then and there. The former is called ex-situ while the latter is termed as in-situ remediation. These techniques could be physical, chemical, biological, or a combination of these. What technique is applied to a polluted area is highly dependent on the topography of the area, environmental and soil conditions, level of contamination, volume of soil to be remediated among others.

Some of the common techniques used for remediation are coagulation-precipitation, adsorption and soil replacement. Coagulation and precipitation work on the principle of neutralizing charges of the particles thus eliminating the repulsive forces acting upon the particles which results in the particles agglomerating eventually leading to precipitation. For arsenic specifically, the soluble arsenic particles are converted to insoluble particles because of the coagulants used and get precipitated. Another common technique is adsorption, which is a process where contaminants are adhered to adsorbent surfaces to which they make contact. This process is a surface phenomenon, so it depends on the surface area:volume ratio of the adsorbent, larger ratios being preferable (Negi and Anand, 1985). It also depends on the adsorbents used, some adsorbents prefer selective ions over others, like activated alumina prefers to adsorb arsenate (US EPA, 2000). Some of the adsorbents used for the remediation of arsenic are activated alumina, granular ferric hydroxide, iron oxide coated sand and activated carbon. The adsorption method is more effective in removing arsenate than arsenite. Therefore, this method works better for samples that contain arsenate predominantly. Both the coagulation-precipitation and adsorption methods require a pre-oxidation treatment and a filtration process after, which decreases efficiency and increases cost, especially in vast areas with high contamination (Sarkar and Paul, 2016). Soil replacement is method in which the polluted soil is either partly or wholly substituted with clean soil to decrease the amount of contamination. However, this technique is only useful in smaller areas and is costly

(Wan et al., 2020). Widespread areas with multiple sources of pollution like mine tailings cannot efficiently be treated with chemical or physical methods. For such areas it is much more feasible, both practically and financially to use biological techniques like phytoremediation and bioremediation.

1.5.2. Phytoremediation

Phytoremediation is a low cost, in situ, method of using plants for environmental cleanup (Salt et al., 1995). It can be done by different means like pollutant stabilization, extraction, degradation, or volatilization. Organic pollutants are usually degraded in the rhizosphere and/or inside the plants. Phytoextraction and phytostabilization are the most common phytoremediation methods to remediate inorganic pollutants. Phytoextraction is a method in which plants extract and accumulate pollutants in their leaves and shoots, and so, are harvested for disposal. This has been used for toxic metals like arsenic and selenium (Blaylock, 2000). Phytostabilization is a process that retains the contaminants either within roots or the rhizosphere to prevent the dispersal of the contaminants further in soil and ground water. This is useful in areas where the arsenic contamination is high as it ensures the contamination is contained in a limited area by limiting the mobility of the contaminants. It also decreases the risk of arsenic entering food chains by decreasing the arsenic from transporting to the above ground plant biomass (Mendez and Maier, 2008; Madejón et al., 2002). Lastly, there is phytovolatilization, in which pollutants are converted into a volatile form and diluted into the atmosphere. For example, *Pteris vittata* is a plant that possesses the ability to volatilize arsenic in the form of methylated arsenic compounds (Sakakibara et al., 2010).

1.5.2.1. General Mechanism of Arsenic Phytoremediation

1.5.2.1.1. Uptake of Arsenic Pollutants

Arsenic, as previously mentioned, can exist in different forms and the mechanism of its uptake depends on its form. Phosphate transporters present in the plants is what leads to uptake of arsenate due to their similar structures. One of the commonly used phosphate transporters are Pi Transporter 1 Family or the PHT1 family (Shi et al., 2004). However, arsenite is taken up by the aquaporin membrane channel proteins such as the Nodule26-like Intrinsic Proteins (NIPs) in plants. For instance, arsenite enters *A. thaliana* through the NIP5;1 and NIP6;1, and *Oryza sativa* through NIP2;1 and NIP 3;2 (Bienert et al.,2008). Some Plasma Membrane Intrinsic Proteins (PIPs) are also permeable to arsenite like the OsPIP2;4 (Mosa et al.,2012). Methylated arsenic species like MMA and DMA also enter through the NIP2;1 membrane channel protein in *O. sativa* (Li et al.,2009).

1.5.2.1.2. Compartmentation and Translocation of Arsenic in Plants

Once As is taken up into plants, there are specific detoxification mechanisms carried out by the plants. The main strategies that plants use to detoxify As are vacuolar sequestration or compartmentation (Verbruggen et al. 2009; Mateo et al. 2019) and translocation (Verbruggen et al. 2009; Fayiga and Saha 2016). For vacuolar sequestration, As(V) is first reduced to As(III) by an arsenate reductase and the As(III) then complexes to phytochelatins so that the complex can be sequestered into the root vacuoles, while some remaining As(III) is effluxed out of the cell by the NIPs (Catarecha et al. 2007; Kamiya et al. 2013; Abbas et al. 2018). The compartmentation of arsenic in root vacuoles is the major mechanism of As phytostabilization.

Translocation of arsenic from the plant roots to and storage of the translocated arsenic in the above ground biomass, such as the shoots and leaves, are the major mechanisms of phytoextraction.

As(V) was first converted to As(III) in the plant roots before being translocated to the leaves via xylem. The As(III) in the leaf cells will be transported into and stored in the vacuoles in form of phytochelatin-As(III) complex (Ma et al. 2001). Arsenic can also be transported from leaves to grains via phloem using the remobilization pathway and this can lead to arsenic accumulation in the grains like rice (Marschner, 2011). Translocation of arsenic is the mechanism by which phytoextraction takes place in plants.

1.5.3. Bacterial Bioremediation

Bacterial conversion between the forms of arsenic highly impacts the mobility of arsenic and its overall toxicity in an area. Living in arsenic contaminated environments, various bacteria have evolved resistant mechanisms to cope with these high levels of arsenic. Some of these arsenic resistant bacteria also develop the ability to oxidize As(III) to As(V) to reduce the toxicity of arsenic and the arsenite oxidizing ability of these bacteria has been utilized for bioremediation of arsenite contaminated soil (Abbas et al., 2018).

1.5.3.1. Arsenic Resistant and Detoxifying Mechanisms of Bacteria

The uptake of arsenite and arsenate by bacteria are processed through different membrane proteins. Since arsenate has a similar molecular structure as phosphate, arsenate can enter a bacterial cell by the phosphate transporter proteins, like the Pit and Pst proteins (Willsky and Malamy 1980; Rosen and Liu 2009). Being a less specific phosphate membrane transporter protein, Pit is more efficient in arsenate uptake than the Pst phosphate transporter. Therefore, in environments containing high concentrations of arsenate, bacteria may shut down the expression of Pit to minimize their arsenic intake (Bertin et al., 2011). Arsenite being structurally similar to glycerol is taken up by bacteria by the glycerol transport system, aquaglyceroporin channel GlpF (Meng et al., 2004).

The arsenite efflux system is the principal arsenite resistant mechanism of arsenic resistant bacteria. The *arsRBC* operon is a cluster of genes responsible for this efflux system (Ji and Silver, 1992; Sofia et al., 1994). The ArsC protein is an arsenate reductase responsible for converting arsenate entering the bacterial cell to arsenite. This arsenite ions together with the other arsenite that enter the cell through the glycerol transport system will be extruded out of the bacterial cell by the arsenite efflux permease (ArsB). The ArsR is an arsenite sensory protein that controls the expression of the *arsB* and *arsC* genes by sensing the presence arsenic concentration in the environment. Since bacteria do not have an arsenate efflux system, arsenate entering the bacterial cells has to be reduced to arsenite before extruded by the arsenite efflux system.

1.5.3.2. Arsenic Oxidation

Despite the fact that arsenic resistant bacteria can protect themselves from both arsenite and arsenate by the ArsRBC efflux system, they do not necessarily reduce the amount of arsenic in the environment. On the contrary, they may increase the amount of arsenite outside themselves by pumping out the arsenite. However, a sub-population of the arsenic resistant bacteria has developed arsenite oxidation systems that reduce the toxicity of arsenite by converting As(III) to As(V).

There are two different bacterial arsenite oxidases, AIO and ARX (Yan et al., 2019). The majority of the As(III) oxidizing bacteria possess the AIO arsenite oxidase. The AIO arsenite-oxidizing bacteria are heterotrophs (i.e., bacteria use organic carbon compounds as energy and carbon sources for growth) and the As(III) oxidation process is a detoxification mechanism of the bacteria (Santini et al., 2000). There is great diversity amongst this group of As oxidizing bacteria as they belong to multiple diverse phyla (Heinrich-Salmeron et al., 2011). The ARX arsenite oxidase is found in chemolithoautotrophic bacteria that use arsenite as an electron donor to generate energy

for growth. They can be aerobic or anaerobic bacteria depending on whether oxygen or nitrate is used as a terminal electron acceptor, respectively (Zargar et al. 2010). For instance, *Rhizobium* sp. strain NT-26 is an aerobic chemolithoautotrophic As(III) oxidizing bacterium (Santini and Vander Hoven, 2004) and *Alkalilimnicola ehrlichii* MLHE-1 is an anaerobic chemolithoautotrophic As(III) oxidizing bacterium (Zargar et al. 2010).

1.6. Plant Growth Promoting Bacteria (PGPB)

A large variety and number of bacteria live in the rhizosphere or root zone of plants. This could be attributed to the presence of sugars, organic acids, amino acids, and other root exudates that support bacterial growth and metabolism (Bais et al., 2006). The interaction of these rhizosphere bacteria could be helpful, damaging, or neutral to the plant (Lynch, 1990). Some of these bacteria show characteristics that can help the plant to grow, be it directly, like by producing plant growth hormones, facilitating in resource procurement or indirectly by competing with undesirable pathogenic microflora present in the soil. These are called Plant Growth Promoting Bacteria or PGPB. Some of the bacterial genera known for their plant growth promoting activities are *Bacillus, Azobacter, Azospirillum, Azoarcus, Arthrobacter, Pseudomonas, Rhizobium, Burkholderia, Gluconacetobacter, Enterobacter, Erwinia* (Murphy et al., 2003, Esitken et al., 2006, Rodríguez and Fraga, 1999).

Production of plant growth hormones like auxins, gibberellins, cytokinins and abscisic acid is one of the more direct ways in which bacteria can help promote plant growth. These compounds can also increase tolerance to abiotic stresses in plants (Asgher et. al, 2015). Auxins are a very important class of phytohormones that stimulate the growth and development by promoting cell differentiation, elongation, and division (Asgher et al., 2015). They also have a very important role

in heavy metal tolerance, such as arsenic (Krishnamurthy and Rathinasabapathi, 2013) and cadmium (Hu et al., 2013).

Another way bacteria can promote plant growth is by decreasing the ethylene level in plants. Ethylene is a chemical which promotes abscission and senescence in plants by inhibiting the synthesis and transport of auxin, in addition to increasing its degradation, thus, lowering diffusible auxin levels (Burg, 1968). Ethylene also increases stress response of plants in soils containing high concentration of heavy metals (Mishra and Kar, 1974; Imsande, 1998). Some PGPB can reduce plant senescence by decreasing ethylene level of plants by hydrolyzing ACC (1-aminocyclopropane-1-carboxylic acid), the precursor of ethylene, using ACC deaminase (Ullah et al., 2015; Glick et al., 1998).

Iron deficiency is common in plants growing in metal contaminated environment and will lead to plant chlorosis (Imsande, 1998). Some PGPB can produce siderophores. These are chemicals that chelate to metals by a complexation reaction and enhance their bioavailability in the rhizosphere. The microbial siderophore-iron complexes are then taken up by plants serving as an iron source (Ma et al., 2011, Bar-Ness et al., 1991).

Phosphate is one of the essential nutrients required for proper growth and development of plant. Even though phosphate is present in soil in abundance, it is mostly adsorbed in the soil matrix or present in its insoluble phosphorous form (or not bioavailable), which cannot support the growth of plants (Khan et al., 2007). This means that phosphorous can often become a limiting factor in development of plants (Feng et al., 2004). It has also been reported that low availability of phosphorus tends to increase the toxic effects of arsenic in plants (Gulz et al., 2005). Hence, solubilization of phosphate is a very important trait that can be found in bacteria like *Pseudomonas striata* and *Burkholderia cepacian* which can promote the growth in plants. Bacteria that have

these plant growth promoting characteristics aid the growth and germination of phytoremediating plants in highly contaminated soils which might otherwise be stunted.

1.7. Enhancing Phytoremediation by PGP As(III)-Oxidizing Bacteria

Arsenic resistant plant growth promoting bacteria (PGPB) are being used to assist growth and germination of phytoremediating plants in a strategy called bacterial assisted phytoremediation. For instance, Ghosh et al. (2011) found *Pseudomonas* sp., *Comamonas* sp. and *Stenotrophomonas* sp. to increase biomass of *Pteris vittata*, which showed its potential to enhance phytoremediation of arsenic contaminated soils. However, these arsenic resistant bacteria did not have the ability to reduce the toxicity of arsenic in the soil. A PGP As(III)-oxidizing bacterium can reduce the arsenic toxicity of soil by converting As(III) to As(V). This will further enhance the growth and remediating ability of phytoremediating plants. Das et al. (2014) isolated eight PGP As(III)-oxidizing bacteria from arsenic contaminated agricultural soils in southwestern Taiwan and showed that they belonged to four diverse bacterial genera. They also showed that a PGP As(III)-oxidizing *Bacillus flexus* strain ASO-6 improved the growth of rice plants in arsenic contaminated soil (Das et al., 2015). To our knowledge, apart from these two studies, there have not been any studies on bacteria that can oxidize arsenic as well as promote plant growth, much less the implications that it might have in arsenic phytoremediation. Therefore, more studies are needed to explore the potential benefits of the PGP As(III)-oxidizing bacteria on phytoremediation to alleviate the toxic effect of arsenic contaminated environments.

2. Rationale, Hypothesis and Objectives

Mining is one of the major causes of arsenic contamination worldwide (Simmler et al., 2016). Northern Ontario is the key mining region of the province, which produces one-third of the mined metal of Canada (Burkhardt et al., 2017). With the increase activities of mining in the region, it is crucial to develop highly effective remediation methods to deal with the contamination problems. Phytoremediation is one of the most widely used methods for cleanup because it is low cost and can be done in-situ (Salt et al., 1995). However, high concentration of arsenic and poor soil conditions at a contaminated site can decrease or inhibit the growth of the plants and reduce the success of phytoremediation. PGP As(III)-oxidizing bacteria have shown potential of improving rice production in arsenic contaminated soil (Das et al., 2014, 2015). In our previous study at a gold mine in Hemlo, Ontario, McFadden (2021) isolated one arsenite oxidizing bacterial strain that can improve the germination and growth of red clover in arsenite contaminated growth media. However, this bacterial strain lacks some of the major plant growth promoting characteristics such as production of plant growth hormones. In addition, only one arsenite oxidizing bacterial strain was isolated after numerous attempts.

The Premier Gold Mine in Beardmore, Ontario was established in 1920s and was in operation until mid-1980s (Ministry of Energy, Northern Development and Mines). The average arsenic concentration of the soil was about 1200 mg/kg at the tailing area of the mine (Table 4.1). Comparing to the soil arsenic concentration (9.85 mg/kg) at the Hemlo gold mine (McFadden, 2021), the Premier Gold Mine is about 120 times higher. It is because of the long history of high arsenic contamination at the Premier Gold Mine, I hypothesize that a diverse and high population of arsenite oxidizing bacteria has evolved at this site. Furthermore, due to the mutualistic interactions between the plants and soil bacteria at the tailing site, the arsenite oxidizing bacteria

in the root zone of the plants in this area have developed certain plant growth promoting characteristics. Finally, these PGP As(III)-oxidizing bacteria can promote the growth of plants in arsenite contaminated conditions.

To test my hypothesis, the objectives of the thesis are:

1. To isolate and characterize the arsenite-oxidizing bacteria in the soil of the tailing site of Premier Gold Mine, Beardmore, Ontario.
2. To determine the plant growth promoting characteristics of these arsenite-oxidizing bacterial isolates.
3. To identify and determine the diversity of these PGP As(III)-oxidizing bacteria isolated from the contaminated site.
4. To determine if the PGP As(III)-oxidizing bacteria improve the germination and growth of *Trifolium pratense*.

3. Materials and Methods

3.1. Soil Sampling and Characterization

Soil was sampled at a tailings site of the Premier Mine, Beardmore, Ontario, Canada (latitude: 49.627693°, longitude: -88.052121°). Soil samples were taken from root zone of plants spread out in the area. They were collected using sterile scoops and bags which were sealed and stored at 4°C until further use. Five soil samples were randomly taken from the site, and chemical analyses were performed for each soil sample with two replicates. In addition, arsenite-oxidizing bacteria were isolated from each soil samples.

Figure 3.1. Map showing the Beardmore mine tailings from which sampling was done.

For the analysis of metals, the soil samples were dried in a drying oven at 105°C for 48 h before digestion. Homogenization of the samples was then done so that they could pass through a 2 mm mesh. Soil samples of 0.2 g aliquots were then allowed to predigest in microwave digestion tubes

in a 3:1 ratio of concentrated HNO_3:HCl acids overnight. They were then digested in a MARS 5 microwave digestion oven (CEM Corporation, NC, USA) at 175°C for 45 min. Samples were then diluted with DDW (double-distilled water) to 40 mL after digestion and the concentrations of Al, As, Ba, Be, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mo, Na, Ni, P, Pb, Sb, Sr, Ti, Va, and Zn were analyzed using ICP-AES (Varian Vista Pro Radial, Varian Inc., Lake Forest, California USA) at the Lakehead University Instrumentation Laboratory (LUIL).A Mettler benchtop SevenMulti dual meter (Mettler Toledo, Mississauga, ON, Canada) was used to measure pH and conductivity of the soil samples in a 1:1 dry soil to DDW ratio.

3.2. Growth Media Preparation

3.2.1. Minimal Salts Growth Media

In 1 L of the minimal salts medium (MSM), which contained 2 g $MgSO_4 \cdot 7H_2O$, 1 g NH_4Cl, 0.505 g K_2HPO_4, 0.05 g $CaCl_2 \cdot H_2O$, 0.0015 g $FeCl_2$, 1 mL trace element solution and 1 mL vitamin solution. The trace element solution was prepared by combining 10 mL of 7.7M HCl, 0.19 g $CoCl_2 \cdot 6H_2O$, 0.1 g $MnCl_2 \cdot 4H_2O$, 0.07 g $ZnCl_2$, 0.006 g H_3BO_3, 0.036 g $Na_2MoO_4 \cdot 2H_2O$, 0.024 g $NiCl_2$ and 0.002 g $CuCl_2$ in DDW to a 1 L final volume. The vitamin solution was prepared by dissolving 50 mg thiamine-HCl, 100 mg pyridoxine-HCl, 50 mg niacinamide, 50 mg Ca D-pantothenate, 1 of cyanocobalamin (B12), 20 mg biotin, 50 mg p-aminobenzoic acid, 50 mg riboflavin, and 20 mg folic acid in 1 L of distilled deionized water (DDW). Other than the vitamin solution, all these solutions were autoclaved for sterilization. The vitamin solution was filter-sterilized with a sterile 0.2 μm polycarbonate filter (Millipore Sigma, Oakville, ON, Canada). The MSM agar was prepared by mixing 15 g granulated agar (Difco, Franklin Lakes, NJ, USA) to 1 L MSM before autoclaving. For the MSM-glucose agar medium (MSMG agar), an extra 0.5% of glucose was added to the MSM agar recipe.

3.2.2. Tryptic Soy Broth (TSB) and Tryptic Soy Agar (TSA)

To prepare Tryptic Soy Broth (TSB), 30 g TSB powder (BD Biosciences, NJ, USA) was dissolved in DDW and autoclaved for sterilization. Tryptic Soy Agar (TSA) was prepared by mixing 15 g granulated agar (Difco, Franklin Lakes, NJ, USA) with 1 L TSB and the TSA was sterilized by autoclaving.

3.2.3. Phosphate Buffer Saline (PBS)

Eight and a half g NaCl, 1.093 g Na_2HPO_4, 0.276 g NaH_2PO_4 were dissolved in DDW and adjusted to pH 7.4 before the solution was topped up to a final volume of 1 L. The PBS solution was sterilized by autoclaving before use.

3.2.4. Luria-Bertani (Miller) Growth Medium (LB)

Twenty-five g of LB (Miller) powder (10 g tryptone, 5 g yeast extract, 10 g sodium chloride; Fisher Scientific, Ottawa, ON, Canada) was added to 1 L of DDW to prepare the LB growth media and then autoclaved for sterilization.

3.2.5. Dworkin and Foster (DF) Medium

To prepare the DF medium (Dworkin and Foster, 1958), 4 g KH_2PO_4, 6 g Na_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 2 g glucose, 2 g citric acid and 0.1 mL of trace element solution (described previously in the composition of Minimal Salts Medium, Section 3.2.1) were dissolved in DDW at pH 7.2 before adjusted to a final volume of 1 L and the growth medium was autoclaved for sterilization before used.

3.2.6. Hoagland's Agar

To prepare the Hoagland's Agar, 1.6 g of Hoagland's No. 2 Basal Salt Mixture (Sigma-Aldrich Canada Co., Oakville, ON, Canada), (which consists of 115.03 mg $NH_4H_2PO_4$, 2.86 mg H_3BO_3, 656.4 mg $Ca(NO_3)_2$, 0.08 mg $CuSO_4 \cdot 5H_2O$, 5.32 mg $Fe_2(C_4H_4O_6)_3 \cdot 2H_2O$, 240.76 mg $MgSO_4$,

1.81 mg $MnCl_2 \cdot 4H_2O$, 0.016 mg MoO_3, 606.6 mg KNO_3, 0.22 mg $ZnSO_4 \cdot 7H_2O$) was added to 1 L DDW. After the salt mixture was completely dissolved, 7.5 g of granulated agar (Difco, Franklin Lakes, NJ, USA) was mixed into the solution and the Hoagland's Agar was autoclaved for sterilization.

3.3. Enrichment of Soil Samples with Arsenite for Isolating Arsenic Oxidizing Bacteria

To increase the population of the arsenite-oxidizing bacteria in the soil samples, two arsenite enrichment steps were performed on the soil. For the first enrichment, 10 g of soil sample (about 5 c.c. in volume) was added to 5 mL of filter-sterilized 20 mM of $NaAsO_2$ (sodium arsenite) and 40 mL of sterile Minimal Salt Medium (MSM, Section 3.2.1) to make the final concentration of arsenite to 2 mM. For Control, 5 mL of 20 mM $NaAsO_2$ was added to 45 mL of MSM without soil. The arsenite-enriched samples were kept for incubation at 25°C and 150 rpm for four weeks. Two mL were taken from each enrichment sample at Week 4 to test for the oxidation of arsenite. For the second arsenite enrichment, 5 mL of each first enrichment sample were added to 40 mL sterile MSM and 5 mL of sterile 20 mM $NaAsO_2$ and the samples were incubated in the same conditions (25°C, 150 rpm) as the first enrichment for four weeks. The soil was also analyzed for arsenite oxidation at weeks 2 and 4.

3.4. Quantitative Analysis of Arsenite Oxidation

In order determine the percentage of As(III) being oxidized to As(V) in a sample, the initial amount of As(III) in a sample was determined. After treatment with the As(III)-oxidizing bacteria, the As(III) and As(V) in the sample were separated by the APDC (ammonium pyrrolidinedithiocarbamate) fractionation protocol. The fractionation protocol separated As(III) and As(V) by chelating the As(III) to APDC. The APDC-As(III) complex was removed from the aqueous sample by carbon tetrachloride (CCl_4) extraction. Both the initial As(III) and the As(V)

concentrations were measured by ICP-AES (ICP-AES Varian Vista Pro Radial, Varian Inc., Lake Forest, CA, USA). As(III) oxidation by the bacteria was determined by calculating the percentage of As(V) converted from the initial amount of As(III) in the sample.

To measure the initial As(III) concentration in the sample, in a 15 mL test tube, 1 mL of 0.1 M sodium acetate (adjusted to pH 5), 1.5 mL DDW and 0.5 mL sample was added. The solution was mixed and 0.5 mL from the test tube was taken and diluted into 11.5 mL of DDW. The diluted sample was then analyzed by ICP-AES for its arsenic concentration (i.e., initial As(III) concentration). To determine the As(V) concentration in the sample after the As(III) oxidation by the bacteria, 0.5 mL of the sample was used for the APDC fractionation. In this process, the 0.5 mL sample was mixed with 1 mL of 0.1 M sodium acetate (adjusted to pH 5), 1.25 mL DDW and 0.25 mL of 1% APDC solution (Sigma, Oakville, ON, Canada) in a 15 mL test tube. The solution was mixed and allowed to react for 5 min. White precipitate formed in the solution indicated the formation of the APDC-As(III) complex. Two mL of CCl_4 were added to the APDC sample solution and the mixture was then shaken for 3 min to extract the APDC-As(III) complex into the organic solvent. The tubes were then centrifuged at 3200 x g for 5 min. Half a mL of the upper aqueous layer was taken and added to 11.5 mL of DDW in a fresh tube. The concentrations of the As(V) in the aqueous phase of the samples were determined by ICP-AES. ICP-AES analysis was carried out by the Lakehead University Instrumentation Laboratory (LUIL).

3.5. Isolation of Arsenite Oxidizing Bacteria

A 10X serial dilution was performed on both the first and second arsenite enrichment soil samples in sterile PBS solution. For the first enrichment samples, the final dilution was 100X and the final dilution of the second enrichment was 1000X. The dilution samples were spread on sterile Minimal Salt Medium with Glucose (MSMG) Agar plates containing 1 mM sodium arsenite ($NaAsO_2$).

Seventy-five mg of cycloheximide was dissolved in 3 mL of 95% ethanol and was diluted with 50 mL of sterile DDW and then added to the MSMG agar to make a final volume of 1 L for the purpose of preventing fungal growth from the enriched soil dilution samples. The plates were then incubated for 72 h at 25°C. A total of 34 colonies were then picked from the agar plates based on different morphologies of the bacterial colonies, two colonies from the 1st enrichment plate and five colonies from the 2nd enrichment plate of each of the five soil samples (except sample 3, for which only 4 from the 2nd enrichment's plate). These 34 isolates were streaked individually on sterile TSA plates for purification and then screened for their arsenite-oxidizing capabilities.

3.6. Screening of Arsenite Oxidizing Bacteria

3.6.1. Qualitative Screening for Arsenite Oxidation (Silver Nitrate Test)

To screen the isolates for their ability to oxidize arsenite, they were grown in sterile TSB containing 1 mM $NaAsO_2$, for 72 h at 25°C. From each bacterial culture, 1.5 ml was pipetted into a sterile 1.5 mL microcentrifuge tube and centrifuged at 8000 x g for 10 min. The supernatant was discarded, and the pellet was washed in 1 mL sterile DDW. It was centrifuged under the same conditions (8000 x g , 10 min) again and supernatant was discarded, and the bacterial pellet was resuspended in 200 µL of sterile DDW. A volume of 20 µL of the washed bacterial sample was mixed with 80 µL of sterile 1 mM As(III) solution and incubated in 25°C for 72 h in a sterile microtiter plate. After the incubation period was over, 100 µL of 0.1 M silver nitrate was added to each well. This was incubated at 25°C in darkness for 24 h. Silver nitrate ($AgNO_3$) reacted with As(V) to form a brown precipitate but remained colourless to pale yellow with As(III).

3.6.2. Quantitative Confirmation of Arsenite Oxidation

Isolates that tested positive in the silver nitrate test were further examined by As(III)/As(V) fractionation and ICP-AES to confirm their ability to oxidize As(III). For this, the isolates were

inoculated in TSB containing 1 mM of sodium arsenite ($NaAsO_2$) and incubated at 25°C and 150 rpm for 72 h. The As(III) and As (V) in the samples was fractionated by APDC and ICP- AES analysis was performed using the same protocol described in the section 3.4.

3.7. Preservation of Bacterial Isolates

Isolates that showed arsenic oxidizing capabilities were grown in TSB overnight and mixed 1:1 with sterile 50% glycerol and were then stored at -80°C. Each sample was preserved in three replicates and stored with date.

3.8. Growth Rates of the Arsenic Oxidizing Isolates

The isolates that were found to oxidize As(III) were grown in sterile TSB at 25°C with 150 rpm shaking. The OD_{600nm} (optical density at 600 nm) of the samples were measured at the time points of 0, 6, 9, 12, 15, 21, and 37 h. The readings obtained were used to plot growth curves of all the 34 isolates. The growth curves were used to determine the growth rate of the bacterial isolates.

3.9. Identification of the As(III)-Oxidizing Bacteria by 16S rDNA Sequencing

3.9.1. DNA Extraction

A modified version of the Chelex extraction protocol by Lambailerie et al (1992) was used to extract genomic DNA of the 34 isolates. The isolates were grown in TSB at 25°C with 150 rpm shaking for 72 h. One mL of each culture was pipetted into a 1.5 mL sterile Eppendorf tube and centrifuged at 20,817 x g for 5 min. The supernatant was discarded, and the pellet was resuspended in 1 mL of sterile DDW and centrifuged again under the same conditions. This washing step was repeated once more and then the supernatant was removed. Ten µL of 10 mg/mL Proteinase K and 200 µL of 5% Chelex in DDW was then added to the tubes. The tubes were mixed by using the vortex and incubated at 55°C in a water bath for 30 min. They were then vortexed again and placed in boiling water for 8 min to inactive the proteinase. The samples were cooled to 37°C in a water

bath. Then, 2 µL of RNase was added to each tube and continued to be incubated at 37°C for 1 h followed by 15 min heating in a 70°C water bath to inactive the RNase. Then, the tubes were centrifuged at 20,817 x g for 5 min and the supernatant samples were transferred to fresh sterile 1.5 mL tubes.

3.9.2. Polymerase Chain Reaction (PCR) for Amplification of a 16S rDNA Fragment

PCR was used to amplify a fragment of the 16S rDNA of the DNA extracted from each isolate. To make the PCR reaction mixture, 31 µL of UV-treated sterilized ddH_2O, 5 µL 2mM dNTP mix (dNTP Master Mix, Fisher Scientific), 5 µL $MgCl_2$ (2mM), 5 µL Taq DNA polymerase buffer, and 1 µL Taq polymerase (1U/µL, Fisher Scientific) was mixed. One µL of each primer (10 µM) was added to the reaction mixture. The primers used were 16S-63F (5'-CAGGCCTAACACATGCAAGTC-3') and 16S-534R (5'-ATTACCGCGGCTGCTGG-3') from Eurofins Genomics, Toronto, Ontario. Finally, 1 µL of extracted genomic DNA was added to the reaction mixture making the final volume to 50 µL. A BioRad MJ Mini Thermal Cycler (Bio-Rad Laboratories Ltd., Mississauga, Ontario, Canada) was used to perform the PCR under the following conditions: 95°C for 5 min, then 34 cycles of 95°C denaturation for 1 minute, 50°C annealing for 1 minute, and 72°C extension for 1 minute, then 10 min of 72°C after the final PCR cycle. The PCR products were kept at 4°C until being used for other analyses.

3.9.3. Gel Electrophoresis

A 1% agarose gel was made by adding 0.5 g of agarose powder (Fisher Scientific) with 50 mL of 1X TAE Buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) and heating for 1.5 min in a microwave so that it was fully dissolved. To this solution, 5 µL of 10 mg/mL ethidium bromide was added while simultaneously stirring to properly homogenize the DNA stain in the agarose. This was gently poured in a cast and left at room temperature to solidify. To each well of the

agarose gel, 10 µL of each PCR product that was mixed with 2 µL of 6X loading dye was added. Five µL of the 0.1 µg/µL GeneRuler 1kb Plus DNA ladder (Fisher Scientific) was used as a reference DNA standard for the electrophoresis process. The samples were then analyzed at 110 V for 20 min in a gel electrophoresis system (Model 200, BioRad). The amplified 16S rDNA fragments in the gel were visualized under UV light using the SynGene Chemigenius Bio Imaging System and GeneSnap computer program.

3.9.4. DNA Sequencing and Phylogenetic Analysis

Purification of the PCR products (i.e., the amplified 16S rDNA fragments) was done using the GeneJET Gel Extraction and DNA Cleanup Micro Kit (Fisher Scientific) according to the manufacturer's protocol. The final volume of the purified PCR product was adjusted to about 10 µL. Out of this, 1 µL was loaded onto a NanoDrop 2000 (Fisher Scientific) to determine the concentration of DNA of each sample. The concentrations of the DNA samples were also confirmed by visualizing the products on a 1% agarose gel using gel electrophoresis under the same conditions as described previously (Section 3.9.3). The concentrations of the purified 16S rDNA fragment samples were adjusted to 20-40 ηg/µL for DNA samples with size between 301-1000 bp, and 40-60 ηg/µL for samples with greater than 1000 bp. Purified PCR products were then sent to Eurofins Genomics (Toronto, Canada) for DNA sequencing. The sequences of the 16S rDNA fragments of the isolates were matched with sequences in the GenBank database to identify the Genus/Species of the arsenite-oxidizing bacteria. The sequence matching analysis was performed by inputting the 16S rDNA sequences to the Basic Local Alignment Search Tool (BLAST) of the National Centre for Biotechnology Information (NCBI). After the arsenite-oxidizing bacteria were identified, the 16S rDNA fragments (each with about 471 bps) of the

bacteria were aligned and analyzed by the MEGA Version X Program (Kumar et al., 2018) to construct a phylogenetic tree of the bacteria using the Maximum Likelihood Method.

A larger fragment of the 16S rDNA (about 1200 bps) of BS2 and BS21 were sequenced to confirm their taxonomic identity because these two arsenite-oxidizing bacterial isolates were chosen to be further studied for their abilities to alleviate arsenite toxicity in plant growth. The primer combinations used to amplify the 16S rDNA samples were 16S-63F/16S-bact806R and 16S-341F/1401R. The amplified DNA fragments were purified and sequenced as described previously. The DNA sequences of the two fragments of each bacterial strain were aligned to obtain a single DNA sequence of about 1200 bp and the single combined sequence was matched with the GenBank database to identify the Genus/Species of the bacterium.

Table 3.1. Primer sequences used to amplify the 16S rDNA of the arsenite-oxidizing bacteria.

Primer	Sequence (5'- 3')	Tm (°C)
16S-63F	CAGGCCTAACACATGCAAGTC	52
16S-534R	ATTACCGCGGCTGCTGGC	55
16S-341F	CCTACGGGRSGCAGCAG	59
16S-1401R	CGGTGTGTACAAGACCC	50
16S-bact806R	GGACTACCAGGGTATCTAATCCTGTT	58

3.10. Plant Growth Promoting Characteristics of the Arsenite-Oxidizing Isolates

3.10.1. Phosphate Solubilization

The arsenite-oxidizing isolates as well as a *Pseudomonas aeruginosa* strain (Barnes et al., 2008) for positive control were grown in sterile TSB at 25°C with 150 rpm shaking for 72 h. National Botanical Research Institute's phosphate growth medium (NBRIP) was made by combining 10 g glucose, 5 g $Ca_3(PO_4)_2$, 5 g $MgCl_2 \cdot 6H_2O$, 0.25 g $MgSO_4 \cdot 7H_2O$, 0.2 g KCl, 0.1 g $(NH_4)_2SO_4$, in 1 L of DDW and the final pH was adjusted to 7 (Mehta and Nautiyal, 2001). The broth was

autoclaved for sterilization before used. Agar plates of the NBRIP growth media was also prepared by adding granulated agar (Difco, Franklin Lakes, NJ, USA) to the NBRI Media before autoclaving the agar growth medium. The isolates were transferred from the TSB cultures to the sterile NBRIP broth and incubated for two days under the same conditions of 25°C and 150 rpm. The isolates and the positive control were then streaked on the sterile NBRIP Plates. Phosphate solubilization was indicated by formation of clear halos on the otherwise opaque plates.

3.10.2. Siderophore Production

All glassware was cleaned with 6M HCl to remove any trace elements and rinsed with DDW before this experiment. To prepare the blue dye, 50 mL solution of 0.06 g of Chrome Azurol S (CAS) (Fluka Chemie AG, Buchs, Switzerland) in DDW was previously prepared and mixed with 9 mL of $FeCl_3.6H_2O$ solution which had been made by dissolving 0.0027 g of $FeCl_3.6H_2O$ in 10 mL of 10 mM HCl. The solution obtained was then combined to 0.073 g of hexadecyltrimethylammonium bromide (HDTMA) dissolved in 40 mL of DDW, producing a blue solution. The CAS-HDTMA solution was autoclaved and stored in a sterile plastic container. For making the Minimal Media 9 (MM9) stock solution, 15 g KH_2PO_4, 25 g NaCl, and 50 g NH_4Cl were dissolved in 500 mL of DDW. Then, 100 ml of the MM9 stock solution were diluted into 750 ml of DDW. To the diluted MM9 stock solution, 32.24g of piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) was dissolved at pH 6 while stirring, and finally adjusted pH of 6.8 using NaOH. To this MM9-PIPES solution, 15 g of agar (Difco, Franklin Lakes, NJ, USA) was added, autoclaved, and cooled to 50°C. Three grams of Casamino Acid was dissolved in 27 mL of DDW, extracted with 3% hydroxyquinoline in chloroform and filter sterilized with a sterile 0.22 µm membrane filter (Polycarbonate filter, Millipore Sigma). Ten ml of filter-sterilized glucose solution (20%, W:V) was also prepared. Once the MM9-PIPES solution mixture was cooled, it

was combined with the Casamino acid and glucose solution. The blue CAS dye was slowly poured along the glass wall while constantly stirring to the liquid MM9-PIPES-Casamino acid agar. Plates were then aseptically poured and allowed to set for 24 h. All the isolates and the positive *Pseudomonas aeruginosa* control were streaked on the plates and incubated at 25°C. Orange halo around bacterial colonies indicate siderophore production by the bacteria (Schwyn and Neilands, 1987).

3.10.3. Production of Indole-3-Acetic Acid (IAA)

The isolates were cultured in sterile LB Medium for 10 h at 25°C and shaking of 150 rpm. After the initial culturing, 1 mL of the isolates were transferred to Erlenmeyer flasks containing 10 ml of sterile LB media supplemented with tryptophan at 0.5 mg/10 mL so that the initial $O.D_{600nm}$ was between 0.05-0.1. Each isolate was then incubated at 25°C with 110 rpm shaking in the dark for 72 h. A volume of 1.5 mL of the cell suspension was collected from each sample after 72 h and centrifuged for 10 min at 8000 x g. One mL of the supernatant obtained was taken and mixed with 2 mL of Salkowski's reagent (37.50 mL 95.5% H_2SO_4, 1.88 mL 0.5 M $FeCl_3 \cdot 6H_2O$ and 60.62 mL distilled H_2O) and allowed to stand at room temperature for 1 h. Reddish pink colour in the solution indicated the production of IAA. The absorbances of the samples were determined at 530 nm using an Ultrospec 1100 Pro spectrophotometer (Biochrom, MA, USA) and the IAA concentrations of the samples were determined by comparing their absorbances to IAA calibration curve (Gordon and Weber, 1951; Oller et al., 2013).

3.10.4. Determining the Presence of 1-Aminocyclopropane-1-Carboxylate (ACC) Deaminase

The isolates were grown in sterile TSB at 25°C and shaking at 150 rpm overnight. One mL of the isolates was taken from the cell cultures and centrifuged at 8000 x g for 10 min. The supernatant

was then removed, and the cell pellet was washed with 1 mL of sterile DF Salts Minimal Medium and centrifuged again. The cells were then resuspended in sterile DF Minimal Medium. For each isolate, three treatments; negative control, positive control, and ACC treatment, were performed in a 96-well microtiter plate. In the negative control treatment, 10 µL of the cell suspension and 190 µL of the DF medium was added. For the positive control treatment, 10 µL of the cell suspension and 190 µL of DF + Nitrogen medium (DF Medium supplemented with a Nitrogen source at a final concentration of 2 g $(NH_4)_2SO_4$/1 L) was mixed, however, in the ACC treatment, 1.5 µL of 0.5 M filter sterilized ACC was added as the sole Nitrogen source to 188.5 µL of sterile DF Medium and 10 µL of the cell suspension (per well). All the treatments had 8 replicates. The microtiter plate was then incubated at 25°C for 72 h. The absorbance was taken at 595nm by a microtiter plate reader (MRX II Microplate Reader, Dynex Technologies, VA, USA). The growth between the three treatments were compared and if the isolates were able to show a significant amount of growth in the ACC treatment, then it showed that the isolates were able to use ACC as the sole nitrogen source for growth which indicated the presence of ACC deaminase (Penrose and Glick, 2003).

3.11. Further Characterization of BS2 and BS21

BS2 and BS21 were selected for further characterization based on their plant growth promoting traits, As(III) oxidation ability and growth rate. They also represented two distantly related branches of the phylogenetic tree constructed by the DNA sequences of the 16S rDNA fragments obtained from the arsenite-oxidizing isolates (Figure 4.7).

3.11.1. Temperature Optimization of BS2 and BS21

BS2 and BS21 were grown in TSB (25°C, 150 rpm) overnight, washed and resuspended in sterile DDW. The cell suspensions were transferred to 50 mL of sterile TSB to achieve an initial cell

density of OD_{600nm} between 0.05- 0.1 and three replicates were set up for each sample. The cultures were then inoculated at four different temperatures: 20°C, 25°C, 30°C, and 35°C in shaker incubators set at 150 rpm. The OD_{600nm} of the samples were measured at 3, 6, 9 and 24 h. The growth curves of each bacterial strain at the four temperatures were compared.

3.11.2. Minimum Inhibitory Concentrations (MIC) of As(III) and As(V) on BS2 and BS21

BS2 and BS21 were grown in sterile TSB at 25°C and 150 rpm overnight. One mL of these were pipetted in sterile 1.5 mL microcentrifuge tubes and centrifuged at 3200 x g for 5 min. The supernatant was discarded, and the cell pellet was resuspended in 1 mL of sterile DDW. This washing step was repeated two more times and then a final cell suspension was made in sterile DDW. In a sterile 96 well microtiter plate, each column of 8 wells were used as replicates and the different isolates were grown in sterile TSB in increasing concentrations of sodium arsenite ($NaAsO_2$) and sodium arsenate (Na_3AsO_4). The concentrations of arsenite used for the two the isolates were 5, 10,11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 and 25 mM along with a positive control of just TSB without arsenite and a negative control without inoculum. The arsenate concentrations used were 200, 400, 500, 520, 550, 570, 590, 600, 620, 640, 660, 680, 700, 720, 740, 760, 780 and 800 mM with a similar setup for the isolates. The plates were incubated at 25°C for 72 h. The sodium arsenite and sodium arsenate stock solutions were filter sterilized with a sterile 0.22 µm filter before using.

3.11.3. Growth and As(III) Oxidation of BS2 and BS21

BS2 and BS21 were grown in sterile TSB with 1mM $NaAsO_2$ at 25°C and 150 rpm until the OD_{600nm} was about 1.0. Each culture was transferred into 50 mL of fresh sterile TSB, which contained 1 mM of $NaAsO_2$, to reach an initial OD_{600nm} of about 0.1. Each isolate had three replicates along with a negative control without inoculum. The samples were incubated at 25°C

and 150 rpm. The optical densities (OD_{600nm}) of the samples were recorded at 3, 6, 9, 24 and 30 h. At each time point, the total arsenic and arsenate concentrations of the samples were determined by As(III)/As(V) fractionation and ICP analysis as described in the Section 3.4.

3.12. Effect of BS2 and BS21 on *T. pratense* Germination and Growth in Growth Medium Contaminated with Arsenite

3.12.1. Seed Sterilization

The red clover seeds were surface sterilized with 90% ethanol for 30 seconds followed by a wash with 25% bleach solution for 5 min and then washed with sterile DDW seven times. They were then left to dry in the dark aseptically in a biosafety cabinet for 24 h.

3.12.2. Inoculum Preparation

BS2 and BS21 were grown in sterile TSB with 0.1 mM As(III) for 48 h at 25°C at 150 rpm. When the cultures reached the OD_{600nm} of 1, 10 mL of the culture was centrifuged at 3200 x g for 5 min and the supernatant was discarded. The pellet was then resuspended in 1 mL of sterile TSB to increase the OD_{600nm} from 1 to 10. This was used as inoculum for the plant experiments.

3.12.3. Planting

Five different concentrations of sodium arsenite ($NaAsO_2$), including a 0 arsenite treatment, were tested on the red clover seeds by diluting a sterile stock solution into the Hoagland's agar medium (Section 3.2.6) to obtain the concentrations: 0, 0.4, 0.6, 1.0, and 1.5 mM. For each arsenite concentration, TSB without bacterial inoculum was considered as control and the treatment was TSB with bacterial inoculum cells. Both control and treatment for each arsenite concentration had three replicates. Ten red clover seeds were planted per container containing the agar medium. Then, 100 µL of inoculum was pipetted on top of each seed for the bacterial inoculum treatment. For the control, 100 µL of sterile TSB was added to the seeds.

The containers were placed in a Sanyo MLR-350 plant growth chamber (Sanyo Electric Co., Ltd., CA, USA) set with a 12-h photoperiod and 12 mW/cm^2 light intensity and temperature of 20°C. The percentage germination of the red clover seeds were determined at day 22. The germinated plants were harvested on day 30. The whole plants (including both shoot and root) were then dried at 70°C for 72 h. The dry weight of the plants was determined and the plant yield of the treatments was calculated.

3.13. Statistics

The statistical analysis was performed using IBM SPSS Version 26. For determining oxidation potential of soil (Section 3.5), an ANOVA was performed between the three treatments using this software. For determining the presence of ACC deaminase (Section 3.10.4), an independent sample t-test was performed between the control and the ACC treatment of each bacterial sample. For determination of statistical significance for temperature optimization for BS2 and BS21 (Section 3.11.1), the one-way analysis of variance (one way ANOVA) was used. For the germination and growth experiments (Section 3.12.3), control treatment was compared with BS2 and BS21 treatments by using 1-way ANOVA. The p value of 0.05 or less indicated statistical significance in all the tests performed. The graphs were plotted using SigmaPlot 12 software integrated with SigmaStat (Systat Software Inc., San Jose California, USA).

4. Results

4.1. Soil Analysis

A summary of the analyses of the soils from the sampling site is given in Table 3.1. In the province of Ontario, the concentration of arsenic in agricultural soils is required to be less than 11 mg/kg, less than 13 μg/L in ground water and less than 6 mg/kg in all types of sediment (Ontario Ministry of the Environment, 2011). The Federal guidelines on inorganic arsenic present in soil is recommended to be less than 12 mg/kg in all agricultural, residential, commercial, and industrial soils (Canadian Council of Ministers of the Environment, 2001). In this study, all the five soil samples at the tailing site of the Premier Mine showed very high concentrations of arsenic when compared to the recommended levels according to the Provincial and Federal guidelines. The average arsenic concentration the tailing was 1208 ± 581.96 mg/kg. Due to the elevated concentration of arsenic in the soil at the site, remediation is necessary to restore the contaminated area.

Table 4.1. Soil chemistry of the Premier Mine tailing site.

Parameter	**Average (± std deviation)***
Conductivity	299 ± 97.70 uS/cm
Nitrogen	0.294 ± 0.27 %
pH	7.56 ± 0.08
Aluminium	5662 ± 2957.63 ug/g
Arsenic	1208 ± 581.96 ug/g
Barium	56 ± 14.68 ug/g
Beryllium	0.25 ± 0.05 ug/g
Calcium	16024 ± 12303.34 ug/g

Cadmium	< 0.2 ug/g
Cobalt	15.8 ± 8.81 ug/g
Chromium	17.38 ± 7.06 ug/g
Copper	40.58 ± 8.33 ug/g
Iron	18072 ± 6456.18 ug/g
Potassium	950 ± 351.28 ug/g
Magnesium	3440 ± 666.48 ug/g
Manganese	971.4 ± 1253.28 ug/g
Molybdenum	< 5 ug/g
Sodium	323.8 ± 132.30 ug/g
Nickel	47.2 ± 25.70 ug/g
Phosphorus	392.6 ± 106.31 ug/g
Lead	26.6 ± 8.66 ug/g
Sulfur	1135.4 ± 575.47 ug/g
Selenium	< 5 ug/g
Silicon	123.8 ± 90.28 ug/g
Strontium	116 ± 51.26 ug/g
Titanium	192.6 ± 195.60 ug/g
Thallium	< 5 ug/g
Vanadium	7.54 ± 4.43 ug/g
Zinc	78.12 ± 34.00 ug/g

* Data represent averages and standard deviation of five soil samples.

4.2. Oxidation Potential of As(III) in the Soil Samples

Five soil samples from the tailing site were enriched with 2mM sodium arsenite to selectively increase the population of arsenite-oxidizing bacteria. The samples were tested for arsenite oxidation (i.e., increase of arsenate) as an indication of the arsenite oxidizing activity of bacteria in the soil. The negative control that had no soil added, no arsenite oxidization was observed in the sample (Figure 4.1). The soil samples showed oxidation of arsenite to arsenate in both the first and second enrichments. Analyses of arsenite oxidation were performed after 4 weeks for the first enrichment and at intervals of 2 and 4 weeks of the second enrichment. As evident from Figure 4.1, both the enrichments show more than 90% oxidation at all time points. The treatments had a p value > 0.05 which implies that the percentage oxidation in all three of enrichment samples were not significantly different from each other. This experiment indicated the presence of a population of highly effective arsenite-oxidizing bacteria in the soil.

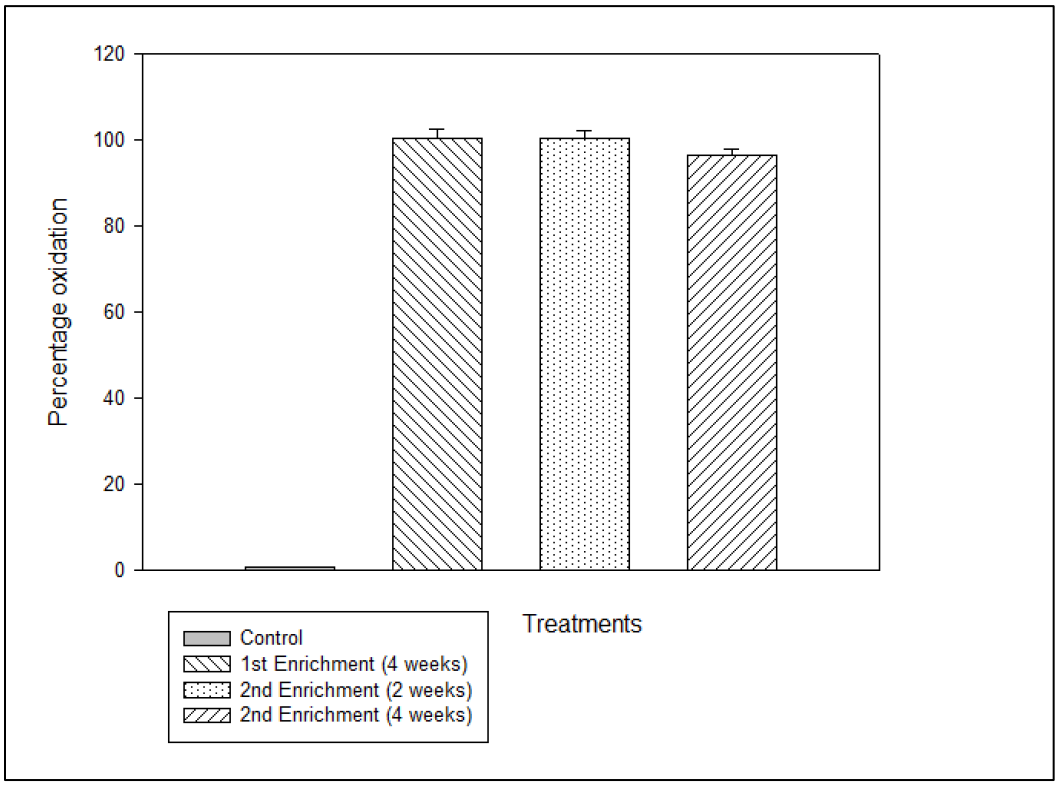

Figure 4.1. As(III) oxidation potential of the soil samples

4.3. Isolation and Screening of Arsenic-Oxidizing Bacteria from the As(III) enriched soil samples

4.3.1. Qualitative Screening for As(III) Oxidation by Silver Nitrate Test

From a 10X serial dilution of the arsenite-enriched soil samples, spread plating was performed on the MSMG agar plates containing 75 µg/mL of cycloheximide and 1 mM $NaAsO_2$. Bacteria were allowed to grow on the MSMG agar plates at 25°C for 72 h. Based on the diverse bacterial colony morphologies observed on the plates, colonies representing various morphologies were picked and streaked on TSA plates. A total of 34 bacterial isolates from the five As(III) enrichment soil samples were isolates and they were screened for their ability to oxidize arsenite using a silver nitrate assay. This is a qualitative test which is based on the principle that in the presence of silver nitrate, arsenite will react with it to form a colorless or yellow color solution whereas arsenate will form a brown precipitate (Figure 4.2). All the isolates showed positive reactions to the silver nitrate screening test with different degree of brown precipitations. This indicated that all the isolates were potential arsenite-oxidizing bacteria with varying degree of ability to oxidize arsenite (Figure 4.3 and Table 4.2).

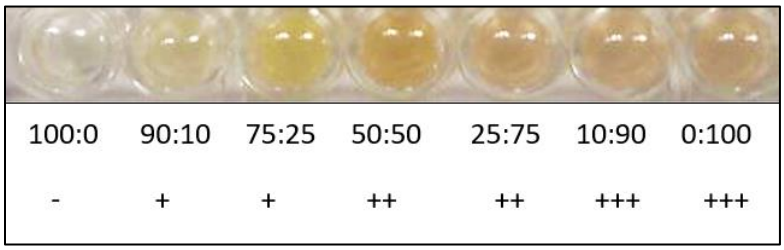

Figure 4.2. Silver nitrate arsenic oxidation test calibration (As(III):As(V))

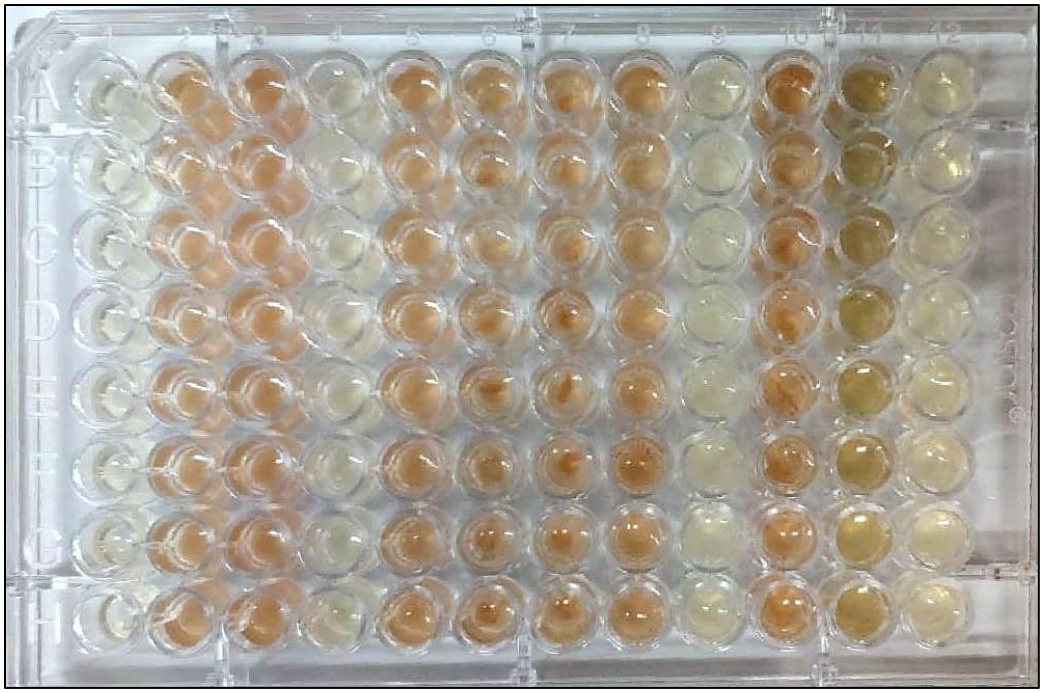

Figure 4.3. Silver nitrate test of 12 of the 34 bacterial isolates (BS1 to BS12, from left to right of the plate).

4.3.2. Quantitative Screening for As(III) Oxidation by As(III)/As(V) Fractionation and ICP-AES

All the isolates were then tested for their ability to oxidize sodium arsenite quantitatively by As(III)/As(V) fractionation and ICP-AES analysis. The Table 4.2 shows the percentage of As(III) oxidized by each individual isolate after 72 h. All the isolates tested were able to oxidize arsenite. However, the ability of the isolates to oxidize arsenite under the same conditions varied among the isolates. The oxidation ability ranged from a low of 21% in BS 7 to a high of 100% in BS 33 and BS 34. The variance in oxidation rates indicated population diversity among the arsenite-oxidizing isolates. Most of the isolates showed good oxidation of As(III) with 91% of them oxidized more than 50% of the arsenite in 72 h. Although the extent of As(III) oxidation shown by the $AgNO_3$ test and the quantitative APDC-ICP assay did not completely agree with each other, it was probably caused by the different set up and conditions of the two assays.

Table 4.2. Percentage oxidation of As(III) to As(V) by the 34 bacterial isolates and the results of silver nitrate experiment.

Isolate	Silver nitrate test*	Percentage oxidation of As(III)
BS 1	+	74.95
BS 2	++	61.58
BS 3	++	80.78
BS 4	+	93.31
BS 5	++	68.16
BS 6	++	88.75
BS 7	++	21.83
BS 8	++	79.45
BS 9	+	78.95
BS 10	++	84.74
BS 11	++	32.64
BS 12	+	81.73
BS 13	+	87.44
BS 14	++	49.89
BS 15	++	82.31
BS 16	+	82.70
BS 17	++	69.11
BS 18	++	63.11
BS 19	++	78.58
BS 20	++	70.66
BS 21	+	86.10
BS 22	+++	60.02

BS 23	+++	52.83
BS 24	+	57.94
BS 25	++	82.13
BS 26	+	72.80
BS 27	+	26.35
BS 28	++	52.27
BS 29	+	76.94
BS 30	+	65.19
BS 31	++	87.39
BS 32	++	93.24
BS 33	+	100.00
BS 34	+	100.00

* The number of "+" suggesting the extent to which arsenite was oxidized when compared with the As(III):As(V) standard ratios shown in Figure 4.2

4.4. Growth Rates of the Isolates

The growth rates of the isolates were calculated, and Table 4.3 shows their doubling time. The growth rates (or doubling time) of the isolates ranged from 1.8 h to 10.7 h. The growth rates of the isolates were very different which hinted at a diverse population of arsenite oxidizing bacteria inhabited in the soil of the tailing site.

Table 4.3. Doubling time of the arsenic-oxidizing isolates

Isolates	**Doubling time (h)**
BS1	2.7
BS2	2.7
BS3	10.7
BS4	5.1

BS5	3.4
BS6	4.1
BS7	7.2
BS8	2.6
BS9	4.4
BS10	4.8
BS11	6.9
BS12	3.4
BS13	7.0
BS14	1.8
BS15	5.0
BS16	4.7
BS17	4.0
BS18	4.1
BS19	5.0
BS20	2.4
BS21	2.9
BS22	4.4
BS23	4.3
BS24	4.9
BS25	2.9
BS26	5.7
BS27	3.1
BS28	7.0
BS29	3.9
BS30	3.4
BS31	6.3
BS32	3.9
BS33	3.9
BS34	5.9

4.5. Plant Growth Promoting Characteristics of the Isolates

4.5.1. Phosphate Solubilization

Most of the phosphates in soils are present in insoluble or unavailable forms and phosphate solubilization makes P uptake easier for plants and helps in their growth. Phosphate solubilisation ability of the bacterial isolates in this study was indicated by the formation of clear halos around their colonies in an otherwise opaque agar plate of NBRI medium (Figure 4.4b). This occurred because of the solubilization of calcium phosphate by bacteria in the NBRI agar plates. LU-71 was used as a negative control (Figure 4.4a). Nineteen out of the 34 isolates were shown to have the ability of solubilizing insoluble phosphate (Table 4.4).

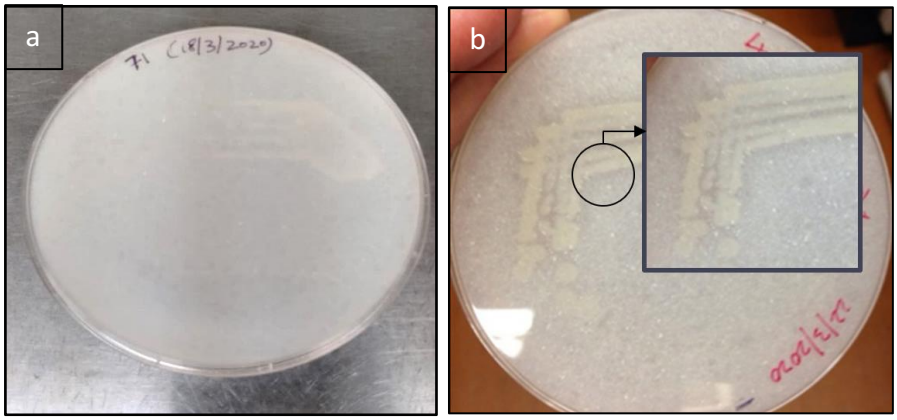

Figure 4.4. Phosphate solubilization assay. LU-71 as a negative control, no halos were observed around the colonies in (a) whereas clear halos are visible around the colonies in (b) with a magnified view of the regions surrounding the colonies.

4.5.2. Siderophore Production

Siderophores are compounds that can chelate strongly to iron(III) and increase its bioavailability to plants thus increasing plant growth. The siderophore test used growth medium that contained Chrome Azurol S (CAS) and HDTMA (hexadecyltrimethylammonium bromide) as indicators. The CAS/HDTMA formed a complex with iron(III) present in the growth medium producing a blue colour, however, a strong iron(III) chelator, like siderophore produced by the isolates, was

able to remove the iron(III) from CAS/HDTMA-Fe(III) complex causing the colour to change from blue to orange (Figure 4.5). The colour change from blue to orange around the bacterial colonies was clearly visible in 32 out of the 34 isolates, indicating the production of siderophores. Only BS8 and BS13 were tested negative for siderophore production (Table 4.4).

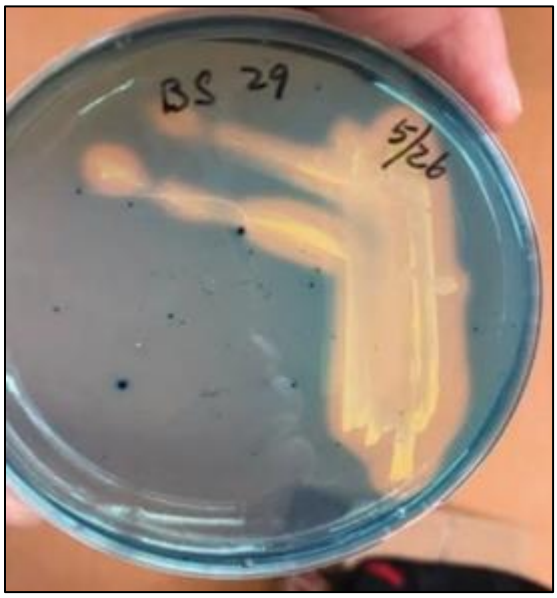

Figure 4.5. Siderophore assay. Orange halos form around bacterial colonies of the isolate BS29 indicating presence of siderophore.

4.5.3. IAA Production

Indole-3-acetic acid (IAA) is an auxin (a plant-growth hormone) that can be produced by some bacteria residing in the rhizosphere of plants and can improve plant growth and development by inducing cell division and elongation. To test the production of IAA, the cell culture of each bacterial isolate was centrifuged and the supernatant was tested with Salkowski's reagent which contained ferric chloride and sulfuric acid which yielded a pink colour upon reaction with IAA (Figure 4.6). The amount of IAA produced by each bacterial isolate was determined by the comparing the absorbance of the sample at 530 nm to an IAA calibration curve. Most of the isolates were tested negative for IAA production. Out of the 34 isolates, only BS2, BS5, BS6, BS8, BS12,

BS17 and BS25 were tested positive. Table 4.4 shows the amount of IAA produced by these IAA-producing isolates.

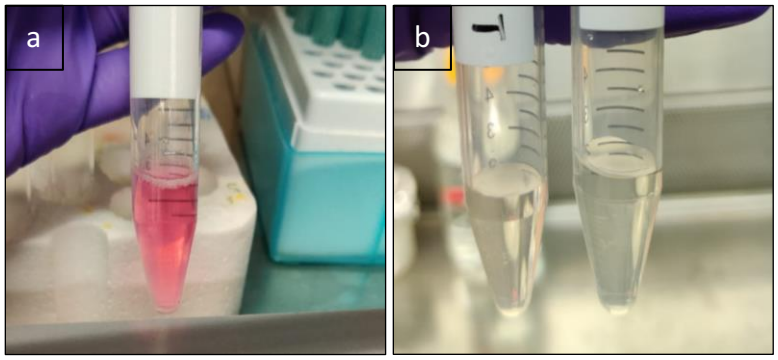

Figure 4.6. IAA assay. Positive samples like BS8 (a) turned pinkish red in colour whereas the negative samples like BS1 (b) stayed colourless.

4.5.4. Presence of ACC Deaminase

Ethylene regulates plant growth by promoting senescence in plants. The presence of ACC deaminase in bacteria helps in lowering ethylene levels in plants by deaminating ACC (1-aminocyclopropane-1-carboxylase), which is the precursor of ethylene. Twenty-five out of the total 34 isolates were able to use ACC as a sole nitrogen source for growth, indicating the production of ACC deaminase by these arsenite-oxidizing isolates (Table 4.4).

Table 4.4. Plant growth promoting characteristics of the As(III)-oxidizing isolates.

Isolate	Phosphate Solubilization	Siderophore Production	IAA production (µg/ml)	Presence of ACC Deaminase
BS1	+	+	-	+
BS2	+	+	23.03 ± 0.04	+
BS3	+	+	-	+
BS4	+	+	-	+
BS5	+	+	10.23 ± 0.04	-
BS6	+	+	4.25 ± 0.01	+
BS7	-	+	-	+
BS8	-	-	30.12 ± 0.01	+

BS9	-	+	-	+
BS10	+	+	-	+
BS11	+	+	-	+
BS12	+	+	5.34 ± 0.04	+
BS13	+	-	-	+
BS14	-	+	-	+
BS15	+	+	-	+
BS16	+	+	-	+
BS17	+	+	3.98 ± 0.03	-
BS18	+	+	-	-
BS19	+	+	-	-
BS20	+	+	-	+
BS21	-	+	-	+
BS22	+	+	-	-
BS23	+	+	-	-
BS24	-	+	-	+
BS25	+	+	6.54 ± 0.01	+
BS26	-	+	-	+
BS27	-	+	-	-
BS28	-	+	-	+
BS29	-	+	-	+
BS30	-	+	-	-
BS31	-	+	-	-
BS32	-	+	-	+
BS33	-	+	-	+
BS34	-	+	-	+

4.6. Identification of the Isolates

The 34 arsenite-oxidizing bacterial isolates were identified by comparing their 16S rDNA fragment sequences to the GenBank database using NCBI BLAST program. Six different genera were identified. Out of the 34 isolates, 22 were *Pseudomonas* spp., 4 *Bacillus* spp., 3 *Variovorax* spp., 3 *Rhodococcus* spp., 1 *Janthinobacterium* sp., and 1 *Nocardioides* sp. (Table 4.5). Based on the DNA sequences of the isolates, a phylogenetic tree was constructed using the maximum likelihood method (Figure 4.7). This showed that the arsenite-oxidizing bacteria in the soil of the tailing site was highly diverse. Among all the isolates, 58% belonged to γ-Proteobacteria, 11.7% to Actinobacteria, 8% to β-Proteobacteria, and 8% to Firmicutes.

Table 4.5. BLAST matches of arsenite-oxidizing bacterial isolates along their percentage similarity and accession numbers.

Isolate	BLAST Match	Percentage Similarity	Accession Number
BS1	*Pseudomonas sp.*	99.76%	FM161427.1
BS2	*Pseudomonas koreensis*	99.45%	MT337499.1
BS3	*Pseudomonas umsongensis*	99.51%	MN758781.1
BS4	*Pseudomonas sp.*	99.33%	MT555393.1
BS5	*Pseudomonas sp.*	99.30%	MT585850.1
BS6	*Pseudomonas sp.*	100.00%	FR775123.1
BS7	*Bacillus sp.*	100.00%	HM566846.1
BS8	*Pseudomonas sp.*	99.53%	FR772204.1
BS9	*Janthinobacterium lividum*	100.00%	CP049828.1
BS10	*Pseudomonas sp.*	99.77%	FR775122.1
BS11	*Pseudomonas plecoglossicida*	94.67%	JX126810.1
BS12	*Pseudomonas sp.*	99.28%	FR775122.1

BS13	*Nocardioides sp.*	97.17%	MT360251.1
BS14	*Pseudomonas sp.*	99.62%	FR775123.1
BS15	*Pseudomonas sp.*	99.77%	MT622198.1
BS16	*Pseudomonas sp.*	99.33%	KC012939.1
BS17	Unidentified (DNA sequencing unsuccessful)	Not applicable	Not applicable
BS18	*Pseudomonas sp.*	99.33%	MT555367.1
BS19	*Pseudomonas sp.*	99.54%	FM161427.1
BS20	*Pseudomonas sp.*	97.52%	FR775123.1
BS21	*Rhodococcus erythropolis*	100.00%	LC552071.1
BS22	*Pseudomonas sp.*	92.44%	MT622198.1
BS23	*Pseudomonas sp.*	98.36%	KJ819611.1
BS24	*Rhodococcus qingshengii*	100.00%	MT632489.1
BS25	*Pseudomonas sp.*	99.35%	FM161478.1
BS26	*Rhodococcus erythropolis*	98.25%	CP050124.1
BS27	*Bacillus aryabhattai*	98.62%	MH010163.1
BS28	*Bacillus paramycoides*	97.02%	MT539151.1
BS29	*Variovorax sp.*	99.33%	MG820625.2
BS30	*Bacillus paramycoides*	98.65%	MT539151.1
BS31	*Variovorax sp.*	98.87%	MG820625.2
BS32	*Variovorax paradoxus*	99.77%	AM285007.1
BS33	*Pseudomonas gessardii*	100.00%	JX514410.1
BS34	*Pseudomonas gessardii*	100.00%	JX514410.1

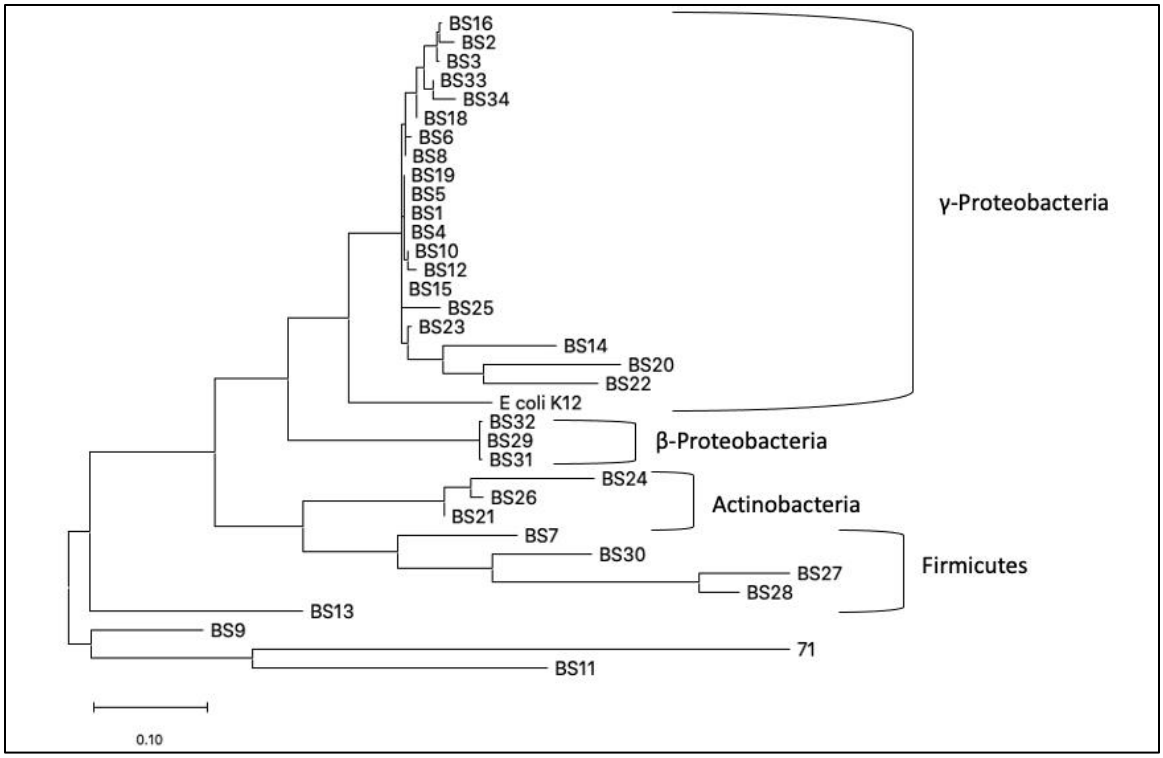

Figure 4.7. The phylogenetic relationship between the arsenite-oxidizing bacterial isolates. For reference purposes, a well-studied model bacterium, *E. coli* strain K-12, and an arsenite-oxidizing isolate, LU-71 (*Caballeronia spp.*), which was previously isolated in our lab were added to this tree. The scale bar at bottom left corner represents the evolutionary distance of the phylogenetic tree.

4.7. Further Characterization of BS2 and BS21

Out of all the isolates, BS2 and BS21 were chosen for further characterization and the plant experiment to test their use to promote plant growth in As(III) contaminated growth medium. These two isolates were chosen because of their As(III)-oxidizing capability, PGP traits, growth rate and diversity. Both isolates oxidized As(III) effectively, and their growth rates were similar. BS2 was a Gram-negative bacterium whereas BS21 is Gram-positive. BS2 showed presence of all the plant growth promoting characteristics that had been tested whereas BS21 only showed the presence of ACC deaminase and production of siderophores.

4.7.1. Further Sequencing and Phylogenetic Analysis of BS2 and BS21

To confirm the identify BS2 and BS21 more accurately, their 16S rDNA were further sequenced. The resulting sequences of the BS2 and BS21 16S rDNA fragments were about 1,200 bps in length and they were closely matched with the 16S rDNA of the *Pseudomonas moraviensis* strain (MN752870.1) and the *Rhodococcus* sp. strain (KX417304.1) with 95.74 and 96.35% similarity, respectively (Table 4.6). Therefore, BS2 was tentatively identified as *Pseudomonas* sp. and BS21 as *Rhodococcus* sp.

Table 4.6. 16S rDNA sequences of BS2 and BS 21 and their closest BLAST matches.

Isolate	Sequence (5'→3')	BLAST match
BS2	GAATGACTTGCTCATGGATTCAGCGGCGGACGGGTGAGTAAT GCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTTCGAAAG GAACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGG ACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTA GCTAGTTGGTGAGGTAATGGCTCACCAAGGCTACGATCCGTA ACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACAC GGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGAC AATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGA AGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGT TGTAGATTAATACTCTGCAATTTTGACGTTACCGACAGAATAA GCACCGGCTAACTCTGTGCCAGCAGCCGCCGGTAATACAGAG GGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGT AGGTGGTTCGTTAAGTTGGATGTGAAATCCCCGGGCTCAACCT GGGAACTGCATCCAAAACTGGCGAGCTAGAGTATGGTAGAGG GTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAG GAAGGAACACCAGTGGCGAAAGCGACCACCTGGACTGATACT GACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTATAT ACCCTGGTAGTCCACACCCTATACGATGTCTACTAACCCTTGG GAGCCCCTAGCTCTCATTGGCGCGCCTCTCGCACTCTGTTGAC ACCCTGGGGAGTGCACCGCCGCGGCGTTAACACTCATATGAG GTGACCGGGGCCCGCACAGGCCGTGGAGCATGTGGTTTAATT CGAAGCAACGCGAACAACCTTACCAGGCCTTGACCTCCAATG AACTTTCCAGAGATGGATTGCTGCCTTCCGGAACATTGAGAC AGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGG GTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTGAGTTACCA GCCCGTTATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAA CCGGAAGAAGGTGGGGATGACCTCGAGT	*Pseudomonas moraviensis* Accession no. MN752870.1 (95.74% similarity)

BS21	ACCCTTATACGTGTTACCCGCTTTCGGACTACGTCGCTGCGGC GCACTCCCTACTGCCGGAAGCCCAACATTTGGAGAAAGTCGT CCCTGCTTCGCGTTCACTGCCATGGACGTCTTCTTCGTGGCCG ATTGATGCACGGTCGTCGGCGCCATTATGCATCCCACGTTCGC AACAGGCCTTAATGACCCGCATTTCTCAAGCATCCGCCAAAC AGCGCAGCAAACACTTTTGGTCGTCGAGTTGACGACCGAACG TCCGCTATGCCCGTCTGAACTCATGACGTCCCCTCTGACCTTA AGGACCACATCGCCACTTTACGCGTCTATAGTCCTCCTTGTGG CCACCGCTTCCGCCCAGAGACCCGTCATTGACTGCGACTCCTT GCTTTCGCACCCATCGCTTGTCCTAATCTATGGGACCATCAGG TGCGGCATTTGCCACCCGCGATCCACACCCAAGGAAGGTGCC TTAGGCACGGCATCGATTGCGTAATTCGCGGGGCGGACCCCT CATGCCGGCGTTCCGATTTTGAGTTTCCTTAACTGCCCCCGGG CGTGTTCGCCGCCTCGTACACCTAATTAAGCTACGTTGCGCTT CTTGGAATGGACCCAAACTGTATATGGCCTTTCGACGTCTCTA CACCGGGGGGGAACACCAGCCATATGTCCACCACGTACCGACA GCAGTCGAGCACAGCACTCTACAACCCAATTCAGGGCGTTGC TCGCGTTGGGGATAGAATACAACGGTCGTGCAATACCACCCC TGAGCATTCTCTGACGGCCCCAGTTGAGCCTCCTTCCACCCCT GCTGCAGTTCAGTAGTACGGGGAATACAGGTCCCGAAGTGTG TACGATGTTACCGGTCATGTCTCCCGACGCTCTGGCACTCCAC CTCGCTTAGGGAATTTCGACCAGAGTCAAGCCTAGCCCCAGA CGTTGAGCTGGGGCACTTCAGCCTCAGCGATCATTAGCGTCTA GTCGT	*Rhodococcus* sp. Accession no. KX417304.1 (96.35% similarity)

4.7.2. Temperature Optimization of BS2 and BS21

The optimal growth temperature for both BS2 and BS21 ranged from 25°C to 30°C when grown in TSB (Figure 4.9a and b). For BS2, it had a similar average growth rate of 4.54 h/generation (between 3 to 9 h) at 25 and 30°C. Furthermore, the growth curves of BS2 at these two temperatures were not significantly different from each other (p value = 0.972). There was little growth of BS2 at 20°C until Hour 9 when it eventually started to grow and almost no growth at 35°C (Figure 4.8a). BS21 reached its optimal rate after 9 h and had an average growth rate of 6.19 h/generation (between 9 to 24 h) at 25 and 30°C. The growth curves of BS21 at 25 and 30°C were

not significantly different from each other (p value = 0.241). The growth of BS21 is much lower at 20°C than 25°C and 30°C, and no growth was observed at 35°C (Figure 4.8b).

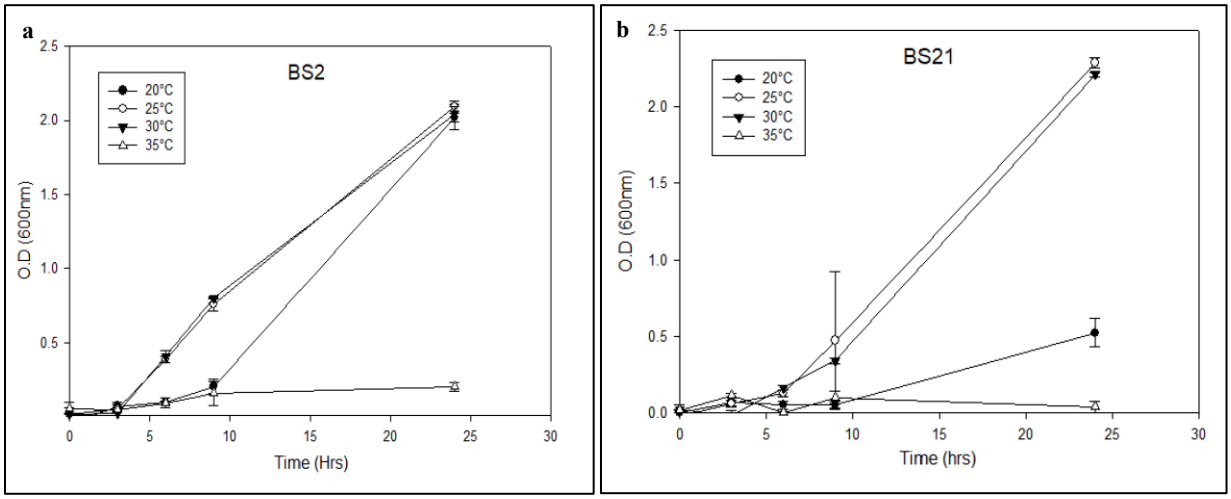

Figure 4.8. The growth of BS2 (a) and BS21(b) at four different temperatures.

4.7.3. Minimum Inhibitory Concentrations of As(III) and As(V)

The minimum inhibitory concentrations (MICs) of BS2 were 21 mM for As(III) and 740 mM for As(V). For BS21, the MICs were 17 mM for As(III) and 570 mM for As(V) (Table 4.7). For BS2, As(III) was about 37 times more toxic than As(V) whereas for BS21 As(III) was about 34 times more toxic than As(V). BS2 was 25 and 31% more tolerance than BS21 in As(III) and As(V), respectively.

Table 4.7. Minimum Inhibitory Concentration (MIC) of sodium arsenite and arsenate of BS2 and BS21.

		Growth (+/-)	
	Concentration (mM)	**BS 2**	**BS 21**
As(III)	*5*	+	+
	10	+	+
	11	+	+
	12	+	+

	13	+	+
	14	+	+
	15	+	+
	16	+	+
	17	+	-
	18	+	-
	19	+	-
	20	+	-
	21	-	-
As(V)	*500*	+	+
	520	+	+
	550	+	+
	570	+	-
	590	+	-
	600	+	-
	620	+	-
	640	+	-
	660	+	-
	680	+	-
	700	+	-
	720	+	-
	740	-	-

4.7.4. Growth and Arsenite Oxidation

Figure 4.9 shows the growth of BS2 and oxidation of arsenite simultaneously. The oxidation of arsenite was below the detection limit (0.8 μM) in the first 3 h. However, arsenite oxidation was detected afterward and 100% oxidation was observed in 30 h when the cell culture reached saturation growth at OD_{600nm} 2.22. Similarly, the oxidation of arsenite was detected after 3 h into

the growth of BS21 (Figure 4.10). The rate of arsenite oxidation was highest between 3 to 6 h and 100% of the arsenite was oxidized as the cells continued to grow to saturation of OD_{600nm} 2.26 in 30 h.

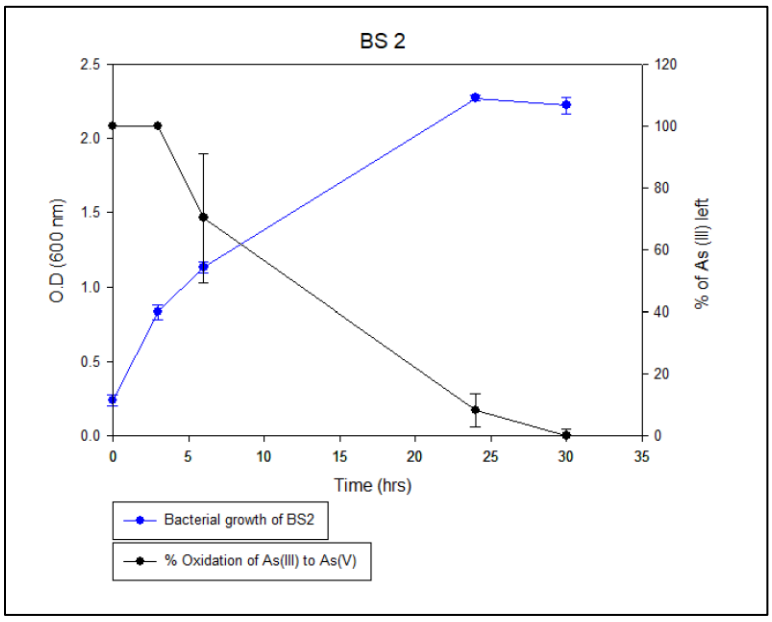

Figure 4.9. Growth of BS2 and the decrease of arsenite as it is oxidized to arsenate over a period of 30 h.

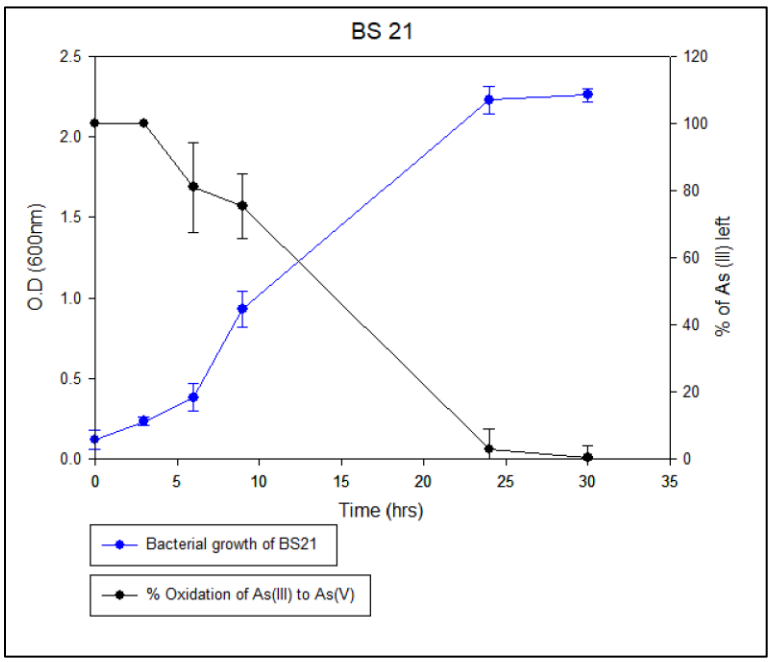

Figure 4.10. Growth of BS21 and the decrease of arsenite as it is oxidized to arsenate over a period of 30 h.

4.8. Effect of BS2 and BS21 on the Growth and Germination of *Trifolium pratense*

4.8.1. Germination of *T. pratense* in Arsenite Media with BS2 and BS21

Germination of *T. pratense* (red clover) was compared with and without the BS2 or BS21 strain when they grew in different concentrations of arsenite. Germination of *T. pratense* seeds in different concentrations of arsenite without any bacterial inoculum were taken as a control treatment. In the control treatment, arsenite significantly decreased the percentage germination of red clover seeds from a concentration of 0.4 mM onwards (Figure 4.11). The percentage germination decreased to zero when the arsenite concentration was increased to 0.6 mM As(III) which can be attributed to the high toxicity of arsenite. There was a significant increase in the germination when the seeds were incubated with inoculum of BS2 ($p = 0.031$) and an average germination rate of 52±13% was observed at arsenite concentrations as high as 1.0 mM. However, seed germination was completely inhibited at 1.5 mM As(III). For BS21, it significantly improved the germination of *T. pratense* at 0.4 and 0.6 mM As(III) ($p < 0.05$). However, seed germination was completely inhibited at 1.0 and 1.5 mM As(III). Furthermore, the beneficial effect of BS2 on the germination of *T. pratense* was significantly great than that of BS21 ($p < 0.05$).

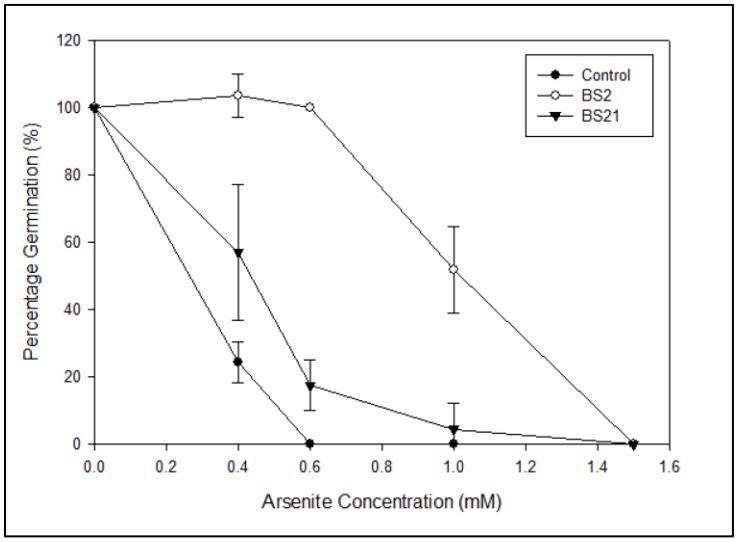

Figure 4.11. Effect of BS2 and BS21 on the germination of *T. pratense* in As(III)-spiked Hoagland's growth medium.

4.8.2. Plant Yield of *T. pratense* in Arsenite Media with BS2 and BS21

The dry weight of *T. pratense* was compared in different concentrations of As(III) spiked growth media when the seeds were incubated with BS2 or BS21 and treatments without bacterial inoculum were used as experimental controls. At 0 mM As(III), the yield was 5.63±0.11 mg/plant in control. The plant yields of the BS2 and BS21 treatments were 5.30±0.34 and 4.89±0.53 mg/plant at 0 mM As(III), respectively, and they were not significantly different to each other and the control treatment. With increase in arsenite concentration, only the BS2 treatment was able to sustain 69, 52 and 35% of the plant growth at 0.4, 0.6 and 1.0 mM As(III), respectively. However, no growth was observed between 0.4 to 1.5 mM As(III) for both the uninoculated and the BS21 treatments (Figure 4.12).

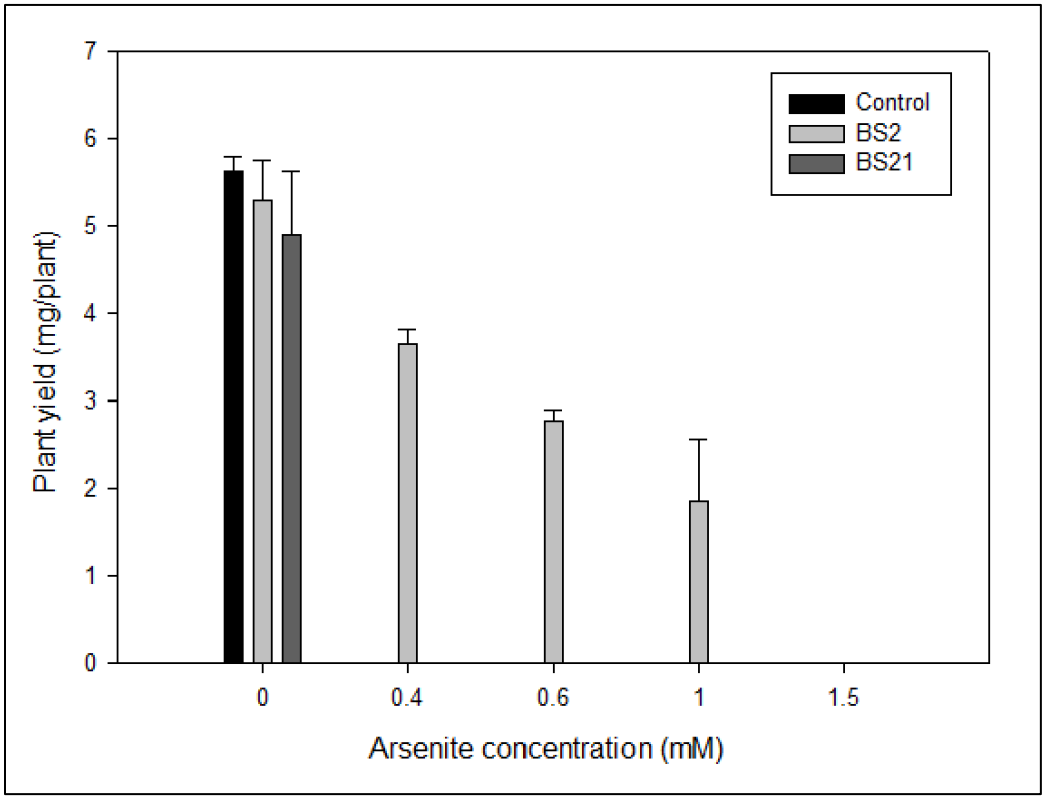

Figure 4.12. Effect of BS2 and BS21 on the growth of *T. pratense* in As(III)-spiked Hoagland's growth medium.

5. Discussion

Remediation is essential to restore arsenic contaminated areas and phytoremediation is one of the strategies that is commonly used. However, toxicity of arsenic in soils can limit growth of phytoremediating plants in such environments. Toxicity of such areas also depends on which form of arsenic is prevalent in the soil, for instance, soil rich in As(III) would cause more harm than As(V) as As(III) can be 60X more toxic than As(V) (Abbas et al., 2018). However, phytoremediation of arsenic can be enhanced by plant growth promoting bacteria (Ghosh et al., 2011). Particularly, bacteria that have the capability to oxidize As(III) to As(V) and produce plant growth promoting factors can be used to increase remediation efforts in highly contaminated areas. Therefore, one of the most important factors that determine the potential of arsenic phytoremediation and detoxification at a site is the presence of a diverse population of PGP As(III)-oxidizing bacteria which also has the ability to promote the growth of phytoremediating plants. Das et al. (2014) showed that the PGP As(III)-oxidizing bacteria isolated from various arsenic contaminated sites in southwestern Taiwan were phylogenetically diverse. However, the diversity of a population of PGP As(III)-oxidizing bacteria within one contaminated site has not been well studied.

Previous studies have demonstrated that presence of arsenite-oxidizing bacteria even in arsenic contaminated soils is rare. This has even led to researchers looking at other sources like sewage (Philips and Taylor, 1976). A study by our lab tested 120 arsenic resistant bacteria isolated from soil collected from a gold mine in Hemlo, Ontario for their ability to oxidize arsenite and only one isolate was tested positive for arsenite oxidation (McFadden, 2021). However, in this study, 100% of the bacterial isolates (34 out of 34 isolates) isolated from the As(III)-enriched soil samples collected from the tailing of Premier Gold Mine were arsenite-oxidizing bacteria. This may be

attributed to the fact that the Hemlo site has a much lower soil arsenic concentration of about 10 mg/kg. For the Premier Gold Mine in Beardmore, the microbial population at the tailing site has been exposed to a high concentration of arsenic (1208 mg/kg) since the mine was established in the 1920s. This condition may drive the evolution of the bacterial population to mitigate the arsenic toxicity by developing an As(III) oxidizing system.

A study conducted by Valverde et al. (2011) discussed the inversely proportional relationship of bacterial diversity and soil arsenic content. In their study, soil with the highest arsenic concentration (943 mg/kg) had the lowest bacterial diversity. However, soils with lower arsenic concentration exhibited higher bacterial diversity. This suggests that soil tested in our study, with an average arsenic concentration of 1208 mg/kg would have less diverse bacterial population. On the contrary, the 34 arsenite-oxidizing bacteria isolates from the Beardmore site were highly diverse. The 16S rDNA sequencing analysis showed that the isolates belonged to six different genera that spread among three diverse phyla (Actinobacteria, Proteobacteria and Firmicutes). Although similar arsenite-oxidizing bacterial species have been identified in other studies (Heinrich-Salmeron et al., 2011), this is the first time such diverse groups of PGP As(III)-oxidizing bacteria were isolated within a single site. Our observation agrees with a metagenomic study by Luo et al. (2014) that the arsenic oxidizing genes were distributed among a wide variety of bacteria within a highly arsenic contaminated site. However, their study was based on the genetic analyses of the DNA extracted from the soil without isolating the bacteria. A possible explanation is the long history of arsenic contamination at the Beardmore site and this allows the PGP As(III)-oxidizing bacteria enough time to evolve to a diverse population.

Free living bacteria isolated from rhizosphere have shown to produce plant growth promoting traits (Ahmad et al., 2008) which is why the soil in this study was collected from the root zone of the

plants growing at the tailing site of the Beardmore gold mine. Each arsenite-oxidizing isolate obtained was tested for four different plant growth promoting traits, including phosphate solubilization, siderophore production, IAA production and ACC deaminase production. Most of the bacteria isolated showed some of the four traits tested which confirmed that bacteria in the rhizosphere may have the ability to promote plant growth, even in an environment with extremely high arsenic concentration. BS2 and BS21 were chosen to test their ability to promote the germination and growth of *T. pratense* in growth medium contaminated with different concentrations of arsenite. They were chosen because they represent two very different groups of bacteria, BS2 being a Gram-negative *Pseudomonas* species whereas BS21 a Gram-positive *Rhodococcus* species. They both had similar As(III)-oxidizing capability and optimal growth rate between 25-30°C. However, BS21 was only positive for siderophore and ACC deaminase production whereas BS2 was positive for all the PGP traits tested. It is of interest to examine the effect of these two genetically diverse arsenite oxidizing bacteria on the growth of plants exposed to As(III) contamination.

One of the most important factors in selecting a plant species for soil remediation is the external factors of the site in question, like climate, soil composition, precipitation among others (Mikalajune and Jasulaityte, 2011). Using common native plants for phytoremediation increases the chances of success of the remediation efforts, decreases plant competition while also ensuring autoecological requirements of plants (Malaval et al., 2010). *T. pratense* or red clover is a commonly found plant in Northwestern Ontario with high biomass production. Its ability to tolerate heavy metals is also an important factor of being used in phytoremediation of mine soils (Davin et al, 2019; Hossner and Hons, 1992; Jin et al, 2013; Mikalajune and Jasulaityte, 2011; Mo et al, 2020). So, *T. pratense* was selected in this study.

Arsenic resistant bacteria isolated from rhizosphere have been studied for their ability to promote plant growth be it in an agricultural environment (Oller et al., 2013) or to assist phytoremediating plants in arsenic contaminated soils (Mesa et al., 2017). However, there have been only two studies exploring the potential of arsenic oxidizing bacteria for their plant growth promoting characteristics. One was done in an agricultural perspective with *Oryza sativa* with the objective of sustainable rice production (Das et al., 2015). In that study, an arsenite-oxidizing bacterial strain that possesses several plant growth promoting traits (including the production of plant growth hormones) was used. They showed that the PGP As(III)-oxidizing strain improved the growth and production of rice plants in arsenic contaminated soil. The second study by Debiec-Andrzejewska et al. (2020) was done in the perspective of improving phytoremediation of arsenic contaminated soil. In their study, an arsenite-oxidizing bacteria without any plant growth promoting traits was used to increase plant growth and arsenic uptake of alfalfa plants successful in an arsenic contaminated soil.

In this study, the abilities of BS2 and BS21 to improve the germination and growth of *T. pratense* in arsenite contaminated growth media were compared in the perspective of improving phytoremediation of arsenic contaminated soil. However, unlike the study of Debiec-Andrzejewska et al. (2020), both BS2 and BS21 possessed certain plant growth promoting traits. Even though both bacterial strains are arsenite oxidizing bacteria, only BS2 produced auxin, a major plant growth hormone. BS2 significantly increased both germination rate and plant yield of *T. pratense* in 0.4, 0.6 and 1.0 mM arsenite. For BS21, despite the improvement in germination, none of the plants inoculated by BS21 grew beyond the seed leaf stage of the seedlings in the arsenite treatments $\geq$ 0.4 mM. The fact that BS21 only improved seed germination but not plant

growth may be attributed several factors. First, seed germination of red clovers may be less sensitive to As(III) toxicity than the growth of their seedlings. Second, the weaker plant growth promoting ability of BS21, such as not able to produce IAA, may not be enough to protect the plant growth at ≥ 0.4 mM As(III). Another reason for the poor performance of BS21 may be attributed to the temperature of the plant experiments. Since the plant experiments was conducted at 20°C, this could affect the growth and metabolism of the bacterial inoculum. Although both bacterial strains did not grow optimally at 20°C, the growth of BS2 adjusted to this temperature and recovered in 9 h. However, the growth of BS21 at this temperature remained slow within 30 h (Figure 4.8). This may be a reason of the sub-optimal plant growth promoting effect of BS21 on the red cover seedlings. Nonetheless, this study demonstrates that many factors could affect the effectiveness of a PGP As(III)-oxidizing bacterium, that include the plant growth promoting traits and physiological characteristics of the bacterium. Furthermore, arsenic concentration, temperature and other environmental factors are also crucial for the success of the PGP As(III)-oxidizing bacteria assisted phytoremediation of arsenic contamination.

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Appendix

Table 1: Sequences of the 33 arsenite-oxidizing isolates.

Isolate	Sequence (5'→3')	BLAST Match
BS1	GTCGGTACGTCAGACACCAACGTATTAGGTTAATGCCCTTCC TCCCAACTTAAAGTGCTTTACAATCCGAAGACCTTCTTCACA CACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTCCAATA TTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCA GTTCCAGTGTGACTGATCATCCTCTCAGACCAGTTACGGATC GTCGCCTTGGTGAGCCATTACCTCACCAACTAGCTAATCCGA CCTAGGCTCATCTGATAGCGCAAGGCCCGAAGGTCCCCTGCT TTCTCCCGTAGGACGTATGCGGTATTAGCGTCCGTTTCCGAG CGTTATCCCCCACTACCAGGCAGATTCCTAGGCATTACTCAC CCGTCCGCCGCTCTCAAGAGAAGCAAGCTTCTCTCTACCGTT CGAC	*Pseudomonas* sp.
BS2	CTGTCGGTACGTCAAATTGCAGAGTATTAATCTACAACCCTT CCTCCCAACTTAAAGTGCTTTACAATCCGAAGACCTTCTTCA CACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTCCAA TATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCT CAGTTCCAGTGTGACTGATCATCCTCTCAGACCAGTTACGGA TCGTAGCCTTGGTGAGCCATTACCTCACCAACTAGCTAATCC GACCTAGGCTCATCTGATAGCGCAAGGCCCGAAGGTCCCCT GCTTTCTCCCGTAGGACGTATGCGGTATTAGCGTTCCTTTCG AAACGTTGTCCCCCACAACCAGGCAGA	*Pseudomonas koreensis*
BS3	AGTATTAATTCTACAGCCCTTCCTCCCAACTTAAAGTGCTTT ACAATCCGAAGACCTTCTTCACACACGCGGCATGGCTGGAT CAGGCTTTCGCCCATTGTCCAATATTCCCCACTGCTGCCTCC CGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGACTGATC ATCCTCTCAGACCAGTTACGGATCGTCGCCTTGGTGAGCCAT TACCTCACCAACTAGCTAATCCGACCTAGGCTCATCTGATAG CGCAAGGCCCGAAGGTCCCCTGCTTTCTCCCGTAGGACGTAT GCGGTATTAGCGTCCCTTTCGAGACGTTGTCCCCCACTACCA GGCAGATTCCTAGGCATTACTCACCCGTCCGCCGCTGAATCA GAGAGCAAGCTCTCTTCATCCGTTCGACTTG	*Pseudomonas umsongensis*
BS4	CTGTCGGTACGTCAGACACCAACGTATTAGGTTAATGCCCTT CCTCCCAACTTAAAGTGCTTTACAATCCGAAGACCTTCTTCA CACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTCCAA TATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCT CAGTTCCAGTGTGACTGATCATCCTCTCAGACCAGTTACGGA TCGTCGCCTTGGTGAGCCATTACCTCACCAACTAGCTAATCC GACCTAGGCTCATCTGATAGCGCAAGGCCCGAAGGTCCCCT GCTTTCTCCCGTAGGACGTATGCGGTATTAGCGTCCGTTTCC GAGCGTTATCCCCCACTACCAGGCAGATTCCTAGGCATTACT CACCCGTCCGCCGCTCGCCACCAGGTACAAGTACCCGTGCTG CCGTTCGACTTGCATGTGTTAGGCCTG	*Pseudomonas* sp.

BS5	TGTCGGTACGTCAGACACCAACGTATTAGGTTAATGCCCTTC CTCCCAACTTAAAGTGCTTTACAATCCGAAGACCTTCTTCAC ACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTCCAAT ATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTC AGTTCCAGTGTGACTGATCATCCTCTCAGACCAGTTACGGAT CGTCGCCTTGGTGAGCCATTACCTCACCAACTAGCTAATCCG ACCTAGGCTCATCTGATAGCGCAAGGCCCGAAGGTCCCCTG CTTTCTCCCGTAGGACGTATGCGGTATTAGCGTCCGTTTCCG AGCGTTATCCCCCACTACCAGGCAGATTCCTAGGCATTACTC ACCCGTCCGCCGCTCGCCACCAGGTACAAGTACCCGTGCTGC CGTTC	*Pseudomonas* sp.
BS6	TGTCGGTACGTCAAACAGATACGTATTAGGTAACTGCCCTTC CTCCCAACTTAAAGTGCTTTACAATCCGAAGACCTTCTTCAC ACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTCCAAT ATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTC AGTTCCAGTGTGACTGATCATCCTCTCAGACCAGTTACGGAT CGTCGCCTTGGTGAGCCATTACCTCACCAACTAGCTAATCCG ACCTAGGCTCATCTGATAGCGCAAGGCCCGAAGGTCCCCTG CTTTCTCCCGTAGGACGTATGCGGTATTAGCGTCCGTTTCCG AGCGTTATCCCCCACTACCAGGCA	*Pseudomonas* sp.
BS7	ACCGTCACCTTGGGAGCAGTTACTCTCCCAAGCATTCTTCCC TGGCAACAGAGCTTTACGATCCGAAAACCTTCATCACTCACG CGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGATTCC CTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCC CAGTGTGGCCGATCACCCTCTCAGGTCGGCTACGCATCGTCG CCTTGGTGAGCCGTTACCTCACCAACTAGCTAATGCGCCGCG GGTCCATCTGTAAGTGATAGATTGCTCCATCTTTCCCGATTC GGTCATGCGACCATATCGCGTATCCGGTATTAGCATTCGTTT CCGAATGTTATCCCAGTCTTACAGGCAGGTTACCCACGTGTT ACTCACCCGTCCGCCGCTAACC	*Bacillus* sp.
BS8	CTGTCGGTACGTCAAACAGATACGTATTAGGTAACTGCCCTT CCTCCCAACTTAAAGTGCTTTACAATCCGAAGACCTTCTTCA CACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTCCAA TATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCT CAGTTCCAGTGTGACTGATCATCCTCTCAGACCAGTTACGGA TCGTCGCCTTGGTGAGCCATTACCTCACCAACTAGCTAATCC GACCTAGGCTCATCTGATAGCGCAAGGCCCGAAGGTCCCCT GCTTTCTCCCGTAGGACGTATGCGGTATTAGCGTCCGTTTCC GAGCGTTATCCCCCACTACCAGGCAGATTCCTAGGCATTACT CACCCGTCCGCCGCTCTCAAGAGAAGCAAGCTTCTCTCTACC GTTCGA	*Pseudomonas* sp.
BS9	GTACCGTCATTAGCAAGAGATATTAGCTCTCACCGTTTCTTC CCTGACAAAAGAGCTTTACAACCCGAAGGCCTTCTTCACTCA CGCGGCATTGCTGGATCAGGCTTTCGCCCATTGTCCAAAATT CCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGT TCCAGTGTGGCTGGTCGTCCTCTCA	*Janthinobacterium lividum*

BS10	CTGTCGGTACGTCAAACACTAACGTATTAGGTTAATGCCCTT CCTCCCAACTTAAAGTGCTTTACAATCCGAAGACCTTCTTCA CACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTCCAA TATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCT CAGTTCCAGTGTGACTGATCATCCTCTCAGACCAGTTACGGA TCGTCGCCTTGGTGAGCCATTACCCCACCAACTAGCTAATCC GACCTAGGCTCATCTGATAGCGCAAGGCCCGAAGGTCCCCT GCTTTCTCCCGTAGGACGTATGCGGTATTAGCGTCCGTTTCC GAGCGTTATCCCCCACTACCAGGCAGATTCCTAGGCATTACT CACCCGTCCGCCGCTCTCAAGAGGTGCAAGCACCTCTCTACC GTTCGACTTGCATGTGTTAGGCCT	*Pseudomonas* sp.
BS11	TTCCTCCCACTTAATGCTTTACAAGACCTTCTTCACACACGC GGCATGGGTGATCTGCTTTCCCCCATTGACCAATATTCCCCA CTGCTGCCTCCATAGATTCGGATTATCTATCTTTAGGTAAGG CTTCCAAG	*Pseudomonas plecoglossicida*
BS12	CTGTCGGTACGTCAAACACTAACGTATTAGGTTAATGCCCTT CCTCCCAACTTAAAGTGCTTTACAATCCGAAGACCTTCTTCA CACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTCCAA TATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCT CAGTTCCAGTGTGACTGATCATCCTCTCAGACCAGTTACGGA TCGTCGCCTTGGTGAGCCATTACCCCACCAACTAGCTAATCC GACCTAGGCTCATCTGATAGCGCAAGGCCCGAAGGTCCCCT GCTTTCTCCCGTAGGACGTATGCGGTATTAGCGTCCGTTTCC GAGCGTTATCCCCCACTACCAGGGAGATTCCTAGGCATTACT CACCCGGCCGCCGCTCTCAAGAGGTGCAANCACCTCTCT	*Pseudomonas* sp.
BS13	CCATCATCGGCGCTTCGTCGGTACTGAAAGAGGTTTACAACC CGAAGGCCGTCATCCCTCACGCGGCGTTGCTGGATCAGGCTT CCGCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGA GTCTGGGCCGTGTCTCAGTCCCGGTGTGGCCGGTCACCCTCT CAGGCCGGCTACCCGTCAAGCCTTGGAAGGGGATTACCCAC CCAGCGANTGAAACAGGCCCCAGATCTTACGGAGGCAACAT GGGGAATATTGGACAATGGGGGGAAGCCTGATCCA	*Nocardioides* sp.
BS14	GTCGGTACGTCAAACAGATACGTATTAGGTAACTGCCCTTCC TCCCAACTTAAAGTGCTTTACAATCCGAAGACCTTCTTCACA CACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTCCAATA TTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCA GTTCCAGTGTGACTGATCATCCTCTCAGACCAGTTACGGATC GTCGCCTTGGTGAGCCATTACCTCACCAACTATCTAATCCGA CCTAGGCTCATCGAAAAC	*Pseudomonas* sp.
BS15	CTTACGTATTAGGTAAATGCCCTTCCTCCCAACTTAAAGTGC TTTACAATCCGAAGACCTTCTTCACACACGCGGCATGGCTGG ATCAGGCTTTCGCCCATTGTCCAATATTCCCCACTGCTGCCT CCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAGTGTGACTGA TCATCCTCTCAGACCAGTTACGGATCGTCGCCTTGGTGAGCC ATTACCTCACCAACTAGCTAATCCGACCTAGGCTCATCTGAT AGCGCAAGGCCCGAAGGTCCCCTGCTTTCTCCCGTAGGACGT	*Pseudomonas* sp.

	ATGCGGTATTAGCGTCCGTTTCCGAGCGTTATCCCCCACTAC CAGGCAGATTCCTAGGCATTACTCACCCGTCCGCCGCTCGCC ACCAGGTACAAGTACCCGTGCTGCCGTTCGACTTGCATGTGT TAGGCCTG	
BS16	TGTCGGTACGTCAAACAGCAAAGTATTAATTTACTGCCCTTC CTCCCAACTTAAAGTGCTTTACAATCCGAAGACCTTCTTCAC ACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTCCAAT ATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTC AGTTCCAGTGTGACTGATCATCCTCTCAGACCAGTTACGGAT CGTCGCCTTGGTGAGCCATTACCTCACCAACTAGCTAATCCG ACCTAGGCTCATCTGATAGCGCAAGGCCCGAAGGTCCCCTG CTTTCTCCCGTAGGACGTATGCGGTATTAGCGTTCCTTTCGA AACGTTGTCCCCCACTACCAGGCAGATTCCTAGGCATTACTC ACCCGTCCGCCGCTGAATCAGAGAGCAAGCTCTCTTCATCCG TTCGACTTGCATGTGTTAGGCCTG	*Pseudomonas* sp.
BS18	TGTCGGTACGTCAAACAGCAAAGTATTAATTTACTGCCCTTC CTCCCAACTTAAAGTGCTTTACAATCCGAAGACCTTCTTCAC ACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTCCAAT ATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTC AGTTCCAGTGTGACTGATCATCCTCTCAGACCAGTTACGGAT CGTCGCCTTGGTGAGCCATTACCTCACCAACTAGCTAATCCG ACCTAGGCTCATCTGATAGCGCAAGGCCCGAAGGTCCCCTG CTTTCTCCCGTAGGACGTATGCGGTATTAGCGTCCGTTTCCG AGCGTTATCCCCCACTACCAGGCAGATTCCTAGGCATTACTC ACCCGTCCGCCGCTCTCAAGAGAAGCAAGCTTCTCTCTACCG TTCGACTTGCATGTGTTAGGCCTG	*Pseudomonas* sp.
BS19	GTCAGACACCAACGTATTTAGGTTAATGCCCTTCCTCCCAAC TTAAAGTGCTTTACAATCCGAAGACCTTCTTCACACACGCGG CATGGCTGGATCAGGCTTTCGCCCATTGTCCAATATTCCCCA CTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCAG TGTGACTGATCATCCTCTCAGACCAGTTACGGATCGTCGCCT TGGTGAGCCATTACCTCACCAACTAGCTAATCCGACCTAGGC TCATCTGATAGCGCAAGGCCCGAAGGTCCCCTGCTTTCTCCC GTAGGACGTATGCGGTATTAGCGTCCGTTTCCGAGCGTTATC CCCCACTACCAGGCAGATTCCTAGGCATTACTCACCCGTCCG CCGCTCTCAAGAGAAGCAAGCTTCTCTCTACCGTTCGACTTG CATGTGTTAGGCCTG	*Pseudomonas* sp.
BS20	CTGTCGGTACGTCAAACAGATACGTATTAGGTAACTGCCCTT CCTCCCAACTTAAAGTGCTTTACAATCCGAAGACCTTCTTCA CACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTCCAA TATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCT CAGTTCCAGTGTGACTGATCATCCTCTCAAACCATTTACGGA TCGCCCCCTTGGTGAGCCTTTACCTCCCAACAAA	*Pseudomonas* sp.

BS21	ACCGTCACTTGCGCTTCGTCCCTGCTGAAAGAGGTTTACAAC CCGAAGGCCGTCATCCCTCACGCGGCGTCGCTGCATCAGGCT TTCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGG AGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGGTCACCCTC TCAGGTCGGCTACCCGTCGTCGCCTTGGTAGGCCATTACCCC ACCAACAAGCTGATAGGCCGCGGGCCCATCCTGCACCGATA AATCTTTCCACCCCAAGTCATGCAACCTGAGGTCATATCCGG TATTAGACCCAGTTTCCCAGGCTTATCCCGAAGTGCAGGGCA	*Rhodococcus erythropolis*
BS22	CACTTACGTATTAGGTAAATGCCCTTCCTCCCAACTTAAAGT GCTTTACAATCCGAAGACCTTCTTCACACACGCGGCATGGCT GGATCAGGCTTTCGCCCATTGTCCAATATTCCCCACTGCTGC CTCCCGTAGGAGTCTGGACCGTGTCTCAGTTCCGGTGGGACT GATCCCCCTCTCACACAAAATACAGATCGCCGCGTTGGGGA GTAATTCCCNCCCCAAATC	*Pseudomonas* sp.
BS23	CTGTCGGTACGTCAAACACTTACGTATTAGGTAAATGCCCTT CCTCCCAACTTAAAGTGCTTTACAATCCGAAGACCTTCTTCA CACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTCCAA TATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCT CAGTTCCAGTGTGACTGATCATCCTCTCAGACCAGTTACGGA TCGTCGCCTTGGTGAGCCATTACCTCACCAACTAGCTAATCC GACCTAGGCTCATCTGATAGCGCAAGGCCCGAAGGTCCCCT GCTTTCTCCCGTANGACGTATGCGGTATTAGCGTCCGTTTCC GAGCGTTATCCCCCGCTACCAGGGGGATTCCTAGGCATTACT CACCCGTCCGCCGCTCGCCACCAGGGACAAGTACCCGTGCT GCCGTTCGA	*Pseudomonas* sp.
BS24	TGCAGGTACCGTCACTTGCGCTTCGTCCCTGCTGAAAGAGGT TTACAACCCGAAGGCCGTCATCCCTCACGCGGCGTCGCTGCA TCAGGCTTTCGCCCATTGTGCAATATTCCCCACTGCTGCCTC CCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGGT CACCCTCTCAGGTCGGCTACCCGTCGTCGCCTTGGTAGGCCA TTACCCCACCAACAAGCTGATA	*Rhodococcus qingshengii*
BS25	TCTGTCGGTACGTCAAAACACTTACGTATTAGGTAAATGCCC TTCCTCCCAACTTAAAGTGCTTTACAATCCGAAGACCTTCTT CACACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTCC AATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGT CTCAGTTCCAGTGTGACTGATCATCCTCTCAGACCAGTTACG GATCGTCGCCTTGGTGAGCCATTACCTCACCAACTAGCTAAT CCGACCTAGGCTCATCTGATAGCGCAAGGGCCGAAGGTCCC CTGTTTTCTCCCG	*Pseudomonas* sp.
BS26	TGCAGGTACCGTCACTTGCGCTTCGTCCCTGCTGAAAGAGGT TTACAACCCGAAGGCCGTCATCCCTCACGCGGCGTCGCTGCA TCAGGCTTTCGCCCATTGTGCAATATTCCCCACTGCTGCCTC CCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCGGTC ACCCTCTCAGGTCGGCTACCCGTCGTCGCCTTGGTAGGCCAT TACCCCACCAACAAGCTGATAGGCCGCGGGCCCATCCTGCA CCGATAAATCTTTCCACCCCAAATCATGCAACCTGAAGTCAT	*Rhodococcus erythropolis*

	ATCCGGTATTAAACCCACTTTCCCAGGCTTATCCCGAAGTGG AAGGCA	
BS27	GGTACCGTCAGGTACGAGCAGTTACTCTCGTACTTGTTCTTC CCTAACAACAGAGTTTTACGACCCGAAAGCCTTCATCACTCA CGCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGATT CCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGT CCCAGTGTGGCCGATCACCCTCTCAGTCGCTATGCATCTTTG CCTTGGAGGGGATGGCCACACTGGGACTGAGACACGGCCCA CACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGG ACGAAAGTCTGACGGAGCACGCCGCGTGAGTGATGAAGGCT TTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACG AGAGTAACTGCTCGTACCTTGACGGTACCTAACCAAAAAGC CAC	*Bacillus aryabhattai*
BS28	GTACCGTCAGGTGCCAGCTTATTCAACTAGCACTTGTTCTTC CCTAACAACAGAGTTTTACGACCCGAAAGCCTTCATCACTCA CGCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGATT CCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGT CCCAGTGTGGCCGATCACCCTCTCANGTCNGCTACGCATCCT TGCCTTGGTGAGGCCCTCCCTCACC	*Bacillus paramycoides*
BS29	CTTACGGTACCGTCATTAGCCCTCTTTATTAGAAAAGGCCGT TTCGTTCCGTACAAAAGCAGTTTACAACCCGAAGGCCTTCAT CCTGCACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTCC AAAATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGT CTCAGTCCCAGTGTGGCTGGTCGTCCTCTCAGACCAGCTACA GATCGAAGGCTTGGTGAGCCTTTACCTCACCAACTACCTAAT CTGCCATCGGCCGCTCCATTCGCGCAAGGTCTTGCGATCCCC TGCTTTCATCCGTAGATCGTATGCGGTATTAGCACAGCTTTC GCTGCGTTATCCCCCACGATTGGGCACGTTCCGATGTATTAC TCACCCGTTCGCCAATCGCCGCCAGGATTGCTCCCGCGCTGC CGTTCGACTTGAATGTGTTAGGCATGA	*Variovorax* sp.
BS30	TAGGTACCGTCAGGTGCCAGCTTATTCAACTAGCACTTGTTC TTCCCTAACAACAGAGTTTTACGACCCGAAAGCCTTCATCAC TCACGCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAAG ATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTC AGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTACGCAT CGTTGCCTTGGTGAGCCGTTACCTCACCAACTAGCTAATGCG ACGCGGGTCCATCCATAAATGACAGCCGAAGCCGCCTTTCA ATTTCCAACCATGCANTTCAAAATGTTATCCGGTATTAGCCC CGGTTTCCCGGAGTTATACCAGACTTATGGGCAGG	*Bacillus paramycoides*

BS31	CTTACGGTACCGTCATTAGCCCTCTTTATTAGAAAAGGCCGT TTCGTTCCGTACAAAAGCAGTTTACAACCCGAAGGCCTTCAT CCTGCACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTCC AAAATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGT CTCAGTCCCAGTGTGGCTGGTCGTCCTCTCAGACCAGCTACA GATCGAAGGCTTGGTGAGCCTTTACCTCACCAACTACCTAAT CTGCCATCGGCCGCTCCATTCGCGCAAGGTCTTGCGATCCCC TGCTTTCATCCGTAGATCGTATGCGGTATTAGCACAGCTTTC GCTGCGTTATCCCCCACAATTGGGCACGTTCCGATGTATTAC TCACCCGTTCGCCACTCGCCGCCAGGATTGCTCCCGCTCTGC CGTTCNACATGCATGTGTTAGGCC	*Variovorax* sp.
BS32	TCTTACGGTACCGTCATTAGCCCTCTTTATTAGAAAAGGCCG TTTCGTTCCGTACAAAAGCAGTTTACAACCCGAAGGCCTTCA TCCTGCACGCGGCATTGCTGGATCAGGCTTTCGCCCATTGTC CAAAATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTG TCTCAGTCCCAGTGTGGCTGGTCGTCCTCTCAGACCAGCTAC AGATCGAAGGCTTGGTGAGCCTTTACCTCACCAACTACCTAA TCTGCCATCGGCCGCTCCATTCGCGCAAGGTCTTGCGATCCC CTGCTTTCATCCGTAGATCGTATGCGGTATTAGCACAGCTTT CGCTGCGTTATCCCCCACGATTGGGCACGTTCCGATGTATTA CTCACCCGTTCGCCACTCGCCCCCAGGATTGCTCCCGCGCTG CCGTTCG	*Variovorax paradoxus*
BS33	CTGTCGGTACGTCAAATTGCAGAGTATTAATCTACAACCCTT CCTCCCAACTTAAAGTGCTTTACAATCCGAAGACCTTCTTCA CACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTCCAA TATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCT CAGTTCCAGTGTGACTGATCATCCTCTCAGACCAGTTACGGA TCGTCGCCTTGGTGAGCCATTACCTCACCAACTAGCTAATCC GACCTAGGCTCATCTGATAGCGCAAGGCCCGAAGGTCCCCT GCTTTCTCCCGTAGGACGTATGCGGTATTAGCGTCCGTTTCC GAACGTTATCCCCC	*Pseudomonas cedrina*
BS34	CTGTCGGTACGTCAAATTGCAGAGTATTAATCTACAACCCTT CCTCCCAACTTAAAGTGCTTTACAATCCGAAGACCTTCTTCA CACACGCGGCATGGCTGGATCAGGCTTTCGCCCATTGTCCAA TATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTCT CAGTTCCAGTGTGACTGATCATCCTCTCAGACCAGTTACGGA TCGTCGCCTTGGTGAGCCATTACCTCACCAACTAGCTAATCC GACCTAGGCTCATCTGATAGCGCAAGGCCCGAAGGTCCCCT GCTTTCTCCCGAAGGACGTATGCGGTATTA	*Pseudomonas cedrina*