The isolation and characterization of arsenic-tolerant endophytes and the potential role of biofilms in arsenic tolerance

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Abstract

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The prevalence and persistence of arsenic pollution is a health and ecological issue of global concern. Conventional engineering techniques (e.g., excavation and capping) have proven be effective at remediating the most highly contaminated sites, but the high cost has limited their use. Phytoremediation presents an opportunity to expand the reach of conventional remediation in a low-cost, environmentally friendly manner. Improving the phytoremediation of arsenic contaminated soils requires reducing phytotoxicity and increasing uptake and translocation of the arsenic into the harvestable tissues. Endophytes, microorganisms colonizing the internal tissues of plants, could provide an effective way to improve phytoremediation outcomes. Beneficial endophytes reduce phytotoxicity in the host plant through improving nutrient acquisition, producing phytohormones, suppressing pathogens, and reducing the host plant stress response. These benefits are well described in the literature, and their effect on plant tolerance has been demonstrated in a wide range of applications. Additionally, endophytes may improve phytoremediation outcomes by directly interacting with the arsenic through various mechanisms such as adsorption, sequestration, chelation, or precipitation.

The endophytes, *Pseudomonas* PD9R and *Rahnella* PD12R, were isolated from native plants growing on arsenic-contaminated soils, and both strains showed tolerance to arsenate (AsV) and

arsenite (AsIII). It was also observed that both strains produced extracellular polymeric substances (EPS) when exposed to arsenic. Biofilms, consisting of microorganisms embedded in a matrix of extracellular polymeric substances (EPS), are a common defense mechanism against various biotic and abiotic stresses, including toxic metals. This investigation looked at the role of endophytic biofilms in arsenic tolerance as a potential direct mechanism by which endophytes might improve phytoremediation outcomes.

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Chapter 1

Microbial endophytes for the clean-up of pollution

This chapter is a book chapter published in *The Good Microbes in Medicine, Food, Biotechnology, Bioremediation, and Agriculture*. It was co-written with Dr. Sharon Doty, who wrote the sections on organic pollutants, while I wrote the sections on inorganic pollutants. It is presented here in its entirety.

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Abstract

Environmental pollution is widespread and poses serious human health risks. The levels of pollution range from extremely toxic in small areas to low levels in expansive areas, with the full range causing harm. Conventional remediation strategies can be effective; however, they are often based on the movement of the contaminant from one area to another, and are prohibitively expensive.

Phytoremediation, the use of plants to clean up pollution, is a low-cost and effective alternative for many sites. But the technology can be limited due to phytotoxic effects of the pollutant, the presence of multiple classes of pollutants, and slow rates of removal. Microbial endophyte-assisted phytoremediation is a relatively new technology that harnesses the power of the plant microbiome, combining the benefits of phytoremediation with those of bioremediation. Increased awareness of this plant-microbe team approach could lead to treatment of many polluted sites currently abandoned due to the high cost of conventional remediation.

Introduction

Pollutants can be classified as either inorganic, such as heavy metals or other elements that cannot be degraded, or organic pollutants that are carbon-based and could be potentially degraded. Conventional methods of remediation include excavation to another site for storage, incineration of the contaminated soil to vaporize the pollutants, or capping of the site to limit public exposure to the contaminants. In the case of polluted water, pump-and-treat is a common conventional method. Alternatively, chemicals are pumped down into the contaminated aquifer to react with the pollutants. All of these methods are expensive and can render the site beyond the financial capability of the site owner for remediation, leaving the public exposed to potential harm.

Phytoremediation, the use of plants to remediate pollutants, offers several advantages over conventional remediation, as it is less expensive, requires lower energy and chemical inputs, enjoys strong public acceptance, and provides a range of ecosystem services, e.g., wildlife habitat, soil stabilization, carbon sequestration, and the potential for secondary market products (McCutcheon & Schnoor 2003; Gerhardt *et al.* 2017). For example, the phytoremediation of moderately contaminated lands using native woody species (e.g., poplar and willow) could be a valuable source of feedstock for the production of bioethanol, or other biochemicals, from cellulosic biomass (Isebrands & Richardson 2014). Ecosystem restoration combined with agroforestry could restore economic productivity to agricultural lands while providing a range of forest ecosystem services.

To maximize the potential of phytoremediation, research is focusing on improving phytoremediation success by addressing the problems of phytotoxicity and the relatively slower rate of remediation, when compared to traditional remediation strategies. One approach is to use genetic engineering of the plants (Dhankher *et al.* 2011; Pilon-Smits & Freeman 2006; Rylott *et*

al. 2015). While this approach has been successful, regulatory hurdles have restricted its deployment. A more practical approach to improvement of phytoremediation has been the partnering of specific microorganisms with plants to overcome some of the limitations of phytoremediation. Endophytes, the microorganisms within plants, have been recognized for providing a wide range of direct and indirect benefits to their host plants (Santoyo *et al.* 2016; Bulgarelli *et al.* 2013), and recent investigations identifying endophytes capable of detoxifying anthropogenic pollutants suggests a potential role in phytoremediation (Doty 2008; Weyens *et al.* 2009a; Weyens *et al.* 2009b; Rylott 2014; Thijs *et al.* 2016).

Since the methods of phytoremediation of inorganic and organic pollutants differ substantially, this review will address the two classes separately.

Organic Pollutants

Background on conventional remediation and phytoremediation of common organic pollutants.

Through intentional dumping or accidental spills, organic pollutants including solvents, polycyclic aromatic hydrocarbons (PAH's), petroleum, and pesticides are common environmental pollutants. Trichloroethylene (TCE), a degreaser and solvent and one of the most common organic pollutants of soil and groundwater, will serve as our example of conventional remediation strategies. TCE is a known human carcinogen (IARC 2012), making its remediation a high priority. Common conventional remediation strategies for TCE include methods to increase its volatilization such as air sparging or pumping and treating through sorption onto barriers (Kuppusamy *et al.* 2016). Averaging between USD $700,000 to $3,000,000 per site, not including annual operating costs (ESTCP 2010), the costs of conventional engineering methods can put

clean-up of a site beyond the financial capacity of the landowner. Another conventional method for remediation of organic pollutants is bioaugmentation or bioremediation (Dangi *et al.* 2019). For bioremediation of TCE, this method usually involves the addition of large quantities of the anaerobic bacterium *Dehalococcoides* that is capable of complete degradation of TCE to harmless ethylene (Vainberg *et al.* 2009). While this strategy can be quite effective, there are numerous challenges in its implementation. The site must first be made anaerobic, usually done through injection of enough carbon sources to cause oxidation of the substrate to deplete available oxygen. The bioremediation strain of *Dehalococcoides* is sensitive to pH, requiring buffers to be added to the site. A major hurdle is that the metabolic pathway can stall at the much more potent carcinogen, vinyl chloride, or at cis-DCE, rendering the site more dangerous. To counter this problem, a strain certified to contain all the genes required for the full metabolic pathway must be used. Effective bioremediation is expensive due to these site preparations and the need for large quantities of the certified strain.

Phytoremediation is an alternative strategy for remediation of TCE that has proven to be successful at numerous sites (Kang 2014). Poplar trees (*Populus* sp.) are metabolically capable of degrading TCE to less toxic forms (Shang *et al.* 2001), and with its extensive root system and ability to take up large volumes of water (Isebrands & Richardson 2014), it is often the tree of choice for phytoremediation of TCE. And since the tree is basically a solar driven "pump and treat" system, it is much less expensive than conventional methods. Major drawbacks, however, are that TCE can have phytotoxic effects, impacting the health and growth of the trees, and that the metabolism of TCE is considered to be too slow (Doty *et al.* 2007). Overexpression of the gene encoding the enzyme in the rate-limiting step in TCE metabolism can greatly enhance degradation

of TCE (Doty *et al.* 2000; Doty *et al.* 2007). However, restrictions on the use of transgenic plants render this solution unusable at this time.

Conventional methods for remediation of polycyclic aromatic hydrocarbons (PAHs), another important class of pollutants, include incineration of the soil or capping of the site to limit human exposure. Despite the hydrophobicity of PAHs, plants have been demonstrated to take up some of these recalcitrant chemicals (Ballach *et al.* 2003; Kuhn *et al.* 2004; Spriggs *et al.* 2005). However, successful phytoremediation of PAHs has been limited due to their high toxicity, causing growth inhibition, reduced transpiration, chlorosis, and wilting of plants (Germaine *et al.* 2009). Similarly, phytoremediation of herbicides, pesticides, explosives, and many other organic pollutants has been hampered by the phytotoxic effects on the plants.

Introduction to endophyte-assisted phytoremediation of organic pollutants

Partnering specific microorganisms and plants can effectively circumvent the problems of direct bioremediation with bacterial strains and of phytoremediation. In this strategy, the plant and bacteria work together, with the plant effectively taking up the pollutant with its wide-spreading roots, and the endophytic bacteria degrading the pollutant within the plant (Doty 2008; Weyens *et al.* 2009b; Khan & Doty 2011), thus reducing the toxicity within the plant and enabling the plant to take up more of the pollutant. With the endophytic bacteria and the plant's own capacity to metabolize the pollutant, the combined effort can effectively degrade the chemicals more fully. Through photosynthesis, the plant can provide the microorganisms with suitable growth substrate since the pollutant itself is generally less than ideal to support bacterial growth. The overall technology is quite simple and adaptable, making it applicable in many settings. For example, the tree species most suited for the specific environment of the contaminated site can be utilized by simply adding the pollutant-degrading endophyte strain to it. Since endophytes generally have a

very broad host range including both broad-leaved eudicots and narrow-leaved monocots such as grasses, they can be inoculated onto most plant species of choice for the site. Since the microorganisms do not need to be engineered to be effective, the technology can be readily utilized without regulatory barriers.

The key to the success of the technology is in isolating the most effective endophyte strains for a particular pollutant. To achieve this challenging goal, we can look to what nature has already selected. Plants can harbor dozens of different bacterial species, and this internal microbial community can be altered according to the environmental conditions (Siciliano *et al.* 2001). Siciliano *et al.* showed that the genes encoding catabolic pathways increased within the root endophyte population in response to the presence of a given pollutant. Since there was plant specificity in the resulting endophytic populations, it seemed that some plant species have the ability to recruit, or selectively expand, the necessary bacteria to detoxify pollutants (Siciliano *et al.* 2001; Siciliano & Germida 1999). Similar research was conducted with endophyte populations of poplar trees growing at a phytoremediation field trial site with BTEX as the major groundwater contaminant (Moore *et al.* 2006). The following sections highlight successes of using plant-microbe partnerships for enhanced remediation of some classes of organic pollutants.

Endophyte-assisted phytoremediation of TCE

A novel endophyte was isolated and characterized from hybrid poplar (*Populus deltoides x nigra* clone DN177) that is resistant to high levels of TCE (Kang *et al.* 2012). Degradation experiments demonstrated that this unique endophyte, identified as PDN3, dechlorinated 80% of TCE in two days without the addition of any inducing phenolic compound. In 24 hours, PDN3 degraded TCE concurrent with a chloride concentration increase with a molar ratio of TCE removal to chloride generation of 1:3, indicating that this endophyte completely degraded TCE.

This was the first report demonstrating that a naturally occurring poplar endophyte can rapidly degrade TCE aerobically without the addition of environmentally harmful inducing substrates. The endophyte strain was successfully deployed at a TCE-contaminated field site (Doty *et al.* 2017). While the uninoculated poplar trees showed signs of TCE phytotoxicity, the trees provided with the endophyte demonstrated superior health and growth. In terms of phytoremediation, the PDN3-inoculated trees had more of the TCE metabolic products than the control (uninoculated) trees. This work represented the first case of endophyte-assisted phytoremediation with trees using a natural endophyte strain. In an earlier study, poplar trees were inoculated *in situ* with the modified endophytic strain containing genes encoding toluene ortho-monooxygenase that co-metabolizes TCE (Weyens *et al.* 2010). Although no increased uptake of TCE was reported, there was a reduction in the phytovolatilization of TCE.

Endophyte-assisted phytoremediation of BTEX, PAHs and petroleum

Another common class of pollutants is BTEX, e.g., Benzene, Toluene, Ethylbenzene and Xylene. In a ground-breaking study by Barac and colleagues, the concept of engineering endophytes for phytoremediation was proven to be successful (Barac *et al.* 2004). The catabolic plasmid from a yellow lupine endophyte was conjugatively transferred to a native endophyte, providing the genes for toluene degradation. The effectively inoculated plants had the ability to significantly reduce the phytovolatilization of toluene, by up to 70%. This research was extended to poplar trees that are more suitable for phytoremediation research (Taghavi *et al.* 2005). Remarkably, it was found that *in planta* horizontal gene transfer occurred such that the plasmid conferring toluene degradation moved directly into the native poplar endophyte population without the need for prior co-culturing.

Research on using endophytes to improve phytoremediation of polycyclic aromatic hydrocarbons (PAHs) has also been quite successful. Using conjugative transfer of a plasmid conferring PAH degradation into an endophytic *Pseudomonas putida* strain, Germaine and colleagues effectively reduced phytotoxicity of naphthalene (Germaine *et al.* 2009). The modified endophyte protected the inoculated peas, resulting in significantly higher germination rates in soil containing the pollutant. Inoculated pea plants had higher transpiration rates in hydroponics containing naphthalene, compared to uninoculated controls, indicating that there was reduced phytotoxicity. In terms of potential phytoremediation improvements, the inoculated plants reduced the amount of naphthalene remaining in soils, removing 37% more of the PAH than the uninoculated pea plants.

In another study, endophyte strains from poplar and willow were screened for the ability to utilize the PAHs as sole carbon and energy sources (Khan *et al.* 2014). The best growing endophyte, termed PD1, was isolated from *Populus deltoides*, and was identified to be a strain of *Pseudomonas putida*. A hydroponics-based experiment was then used to assess the protective effect of PD1 strain against the phytotoxicity of phenanthrene on willow. Results after 3 weeks of exposure to the toxin indicated that PD1 provided strong protective effects to the plants in the pollutant-saturated solution. There was an increase in root and shoot lengths and an overall increase in the biomass of the inoculated plants compared to the uninoculated plants. In the uninoculated control plants, the roots turned brown, and the leaves fell off, rendering these plants ineffective for phytoremediation. The endophyte-inoculated plants were able to remove more of the phenanthrene from solution, and in a soil experiment with willows and grasses, inoculated plants removed up to 40% more of the phenanthrene. Following the success of the lab studies, the endophyte strain was deployed at a PAH-contaminated field site (Landmeyer *et al.* 2020). The

inoculated trees were able to grow larger than the control trees despite the high concentration of the pollutant, indicating that the strain helped reduce the phytotoxic impacts.

Phytoremediation of petroleum can also be enhanced with the help of microorganisms. For example, grasses able to grow well in crude oil-contaminated soil harbor hydrocarbon-degrading endophytes (Fatima *et al.* 2015). Addition of an endophytic consortium consisting of two of these strains resulted in 78% crude oil remediation in the inoculated grasses compared to up to 66% for the uninoculated plants (Fatima *et al.* 2016). Importantly, the uninoculated plants had reduced growth in the contaminated soils compared to uncontaminated soils whereas the endophyte-inoculated plants showed increased growth despite the pollutant. While the majority of research papers on bioaugmentation for improved phytoremediation of petroleum focus on grasses, trees could also likely benefit from endophyte inoculations. Poplar trees have been used for phytoremediation of soils contaminated petroleum hydrocarbons, even under environmentally challenging conditions such as in the boreal forests of Scandinavia (Lopez-Echartea *et al.* 2020). In a study comparing 20 poplar clones and two willow clones and different sizes, the survival rate in soils heavily contaminated with hydrocarbons ranged from 26-100%, with large (60cm) poplar stakes of commercial clones performing especially well (Zalesny *et al.* 2005). Since poplar and willow are propagated through cuttings rather than seeds, they harbor a wealth of well-established endophytes. More research needs to be conducted on the endophytes within these commercial clones that are used primarily for phytoremediation projects.

Endophyte-assisted phytoremediation of herbicides and pesticides

One of the first lab examples of the effectiveness of endophyte-assisted phytoremediation was on the herbicide 2,4-dichlorophenoxyacetic acid, commonly known as 2,4-D. In this ground-breaking study, Germaine and colleagues inoculated pea plants with a genetically tagged poplar

endophyte that naturally possessed the ability to degrade 2,4-D (Germaine *et al.* 2006). Compared to uninoculated plants, the inoculated plants showed a higher capacity for herbicide removal from soil and yet showed no 2,4-D accumulation in their aerial tissues, indicating that the herbicide was completely degraded within the plants by the poplar endophyte.

Chlorpyrifos is a common broad-spectrum organophosphorus pesticide used worldwide. Though it is used against agricultural pests, it also kills beneficial arthropods including pollinator bees (Cox 1995). Chlorpyrifos has major human health impacts, interfering with the nervous system (US EPA). Phytoremediation can be used to help remove chlorpyrifos in constructed wetlands designed to capture the run-off from agricultural fields (Moore *et al.* 2002). Riparian buffers which are planting strips to protect streams from agricultural chemicals could be another way to absorb the organophosphorus pesticide. The riparian species, poplar and willow, were tested in hydroponics experiments and were shown to take up and fully degrade chlorpyrifos over several weeks (Lee *et al.* 2012). Some bacteria are able to degrade the pesticide and may offer a mechanism to increase the speed and effectiveness of phytoremediation of this important pollutant. For examples, the root endophyte *Pseudomonas* strain BF1-3 can hydrolyze the organophosphorus pesticide (Barman *et al.* 2014), ryegrass inoculated with a chlorpyrifos-degrading endophyte, *Mesorhizobium* sp. strain HN3, improved plant health and degradation of the pesticide within the plant (Jabeen *et al.* 2016).

Endophyte-assisted phytoremediation of more recalcitrant organic pollutants

Recalcitrant pollutants are those that are especially difficult to remediate. An important mechanism by which microorganisms could help improve phytoremediation of recalcitrant organic pollutants is through production of surfactants that release the chemicals bound tightly to soil particles, thus allowing for plant uptake of the pollutant (Wenzel 2009; Feng *et al.* 2017; Li *et al.*

2012; Soleimani *et al.* 2010). Surfactants can be added directly to contaminated soils; however, they can be costly, not only monetarily but environmentally. If a rain event occurs too soon after surfactants are administered, for example, the newly released pollutant can more readily leach into the aquifer or surrounding aquatic ecosystems. Precise calibration of the amount of surfactant to add to soils to release the pollutant for plant uptake without rendering the site phytotoxic is another challenge. Using biosurfactant-producing microorganisms, on the other hand, could allow for a small but steady amount of the pollutant to be released, improving plant uptake of the chemical at a rate it can metabolize without harm. Indeed, addition of consortia of strains specifically chosen for strong biosurfactant production did successfully improve phytoremediation of hydrophobic organic pollutants (Pathak & Keharia 2014).

A class of pollutants that is particularly recalcitrant to remediation are the polychlorinated biphenyls (PCB's) which are hydrophobic, chemically stable, and are characterized as persistent organic pollutants (POP's). PCB's bind tightly to soil, rendering plant uptake of these pollutants challenging (Aken *et al.* 2010). Since metabolism of PCB's is energetically expensive, additional carbon sources are needed to support microbial growth, thus lending itself well to the use of plant-microbe partnerships for effective remediation. Genes required for PCB degradation can be transferred from PCB-degrading bacteria into bacterial strains capable of efficient colonization of plant roots (Aken *et al.* 2010). Several research groups successfully engineered strains in this way, resulting in improved remediation of this important class of toxic pollutants (Brazil *et al.* 1995; Villacieros *et al.* 2005).

An emerging area of concern is the prevalence of microplastics in the environment. In agriculture, sources of microplastics include insulating plastic films, controlled-release chemical fertilizer capsules, and biosolids applications (Song *et al.* 2018; Horton *et al.* 2017; Song *et al.*

2020). These microplastics can be absorbed by plants; therefore, phytoremediation of this type of pollutant is a possibility. Plastic-degrading microorganisms could potentially be harnessed and partnered with appropriate plants. Some of the bacterial strains with the ability to degrade plastics such as polyvinyl chloride and polystyrene are Pseudomonas species that can associate with plants. Endophyte-assisted phytoremediation of microplastics will be an important new technology to develop.

Inorganic Pollutants

Background on inorganic pollutants

A legacy of industrial, manufacturing, and agricultural activities has resulted in widespread environmental contamination in both urban and rural areas. Because of their persistence in the environment and toxicity at low levels, the heavy-metals and metalloids (collectively referred to here as HM), e.g., cadmium (Cd), lead (Pb), mercury (Hg), and arsenic (As), are of particular concern. Unlike organic pollutants, which can be entirely or partially detoxified by various microorganisms, HM are non-degradable and can only be transformed into less toxic and/or less bioavailable forms. This means that HM pollution accumulates in the environment, representing a serious, long-term threat to human health and ecosystems.

Generally, metallic chemical elements with a relatively high density and that are toxic at low concentrations are classified under the broader term heavy metals (Burd *et al.* 2000; Ma *et al.* 2011). HM are widely distributed in the Earth's crust, although typically at low concentrations. They enter the environment via the natural weathering of volcanic, sedimentary, and metamorphic rocks, and the biochemical activities of microbes, and are then dispersed via the erosive forces of wind and water. While natural hot spots of some HM do occur, these natural processes are often

exacerbated as a result of human activities that alter environmental conditions, e.g., exposure of HM bearing ores in mining operations, or the over-extraction of groundwaters for agriculture (Yuan *et al.* 2014).

Whether through incidental emissions or intentional applications, elevated levels of HM occur in both urban and rural environments as a result of a wide range of activities, such as mining and smelting operations, the consumption of fossil fuels, applications of agrochemicals, manufacture of electronics, the production and consumption of consumer, industrial, and military products, and the improper disposal of solid wastes and wastewaters (Yuan *et al.* 2014). Public consumption of modern electronic devices and the growing markets for rechargeable batteries will continue to drive the demand for HM (Yang & Hoffman 1984; Wang *et al.* 2002).

Globally, it is estimated there are more than 5 million sites covering 100 million hectares of land contaminated by various HM, and the risk of public exposure is increasing with urbanization (Burd *et al.* 2000). In China nearly 20% of the agricultural land is believed to be affected by HM, while the European Union reports more than 200,000 sites as contaminated with HM (Burd *et al.* 2000). In the United States, the Environmental Protection Agency lists more than 500,000 sites at least moderately contaminated with HM, with an additional 1,327 sites listed under the Superfund program (EPA, 2019).

Humans are exposed to HM through food, water, air, and soil, which enter the body via inhalation, ingestion, and dermal contact, acting as systemic toxins that are associated with different neurotoxic, carcinogenic, mutagenic, teratogenic, cardiotoxic effects (Sheng *et al.* 2008; Ma *et al.* 2009; Zhang *et al.* 2011). While all HM are toxic at certain thresholds, some do have a physiological role. For example, the essential HM copper (Cu), iron (Fe), manganese (Mn), and zinc (Zn) are required for biochemical processes, and are non-toxic below certain thresholds,

where the non-essential HM, such as Cd, Pb, Hg, and As, have no known biological function and are toxic at trace levels for most organisms (Zhang *et al.* 2011).

Phytoremediation of inorganic pollutants

Because they cannot be degraded, the phytoremediation of HM requires specific strategies that differ from those used for remediation of organic pollutants. While the approach depends on the nature of the pollutant, the site conditions, and type of plant(s) used, the most common strategies can be categorized as: phytoextraction, phytofiltration, and phytostabilization (Salt *et al.* 1995). Phytoextraction, and phytofiltration in aquatic systems, involves the uptake of contaminants by the roots, followed by the translocation and accumulation in the harvestable tissues, while phytostabilization sequesters HM in and around the roots by converting the metals to less bioavailable forms, preventing mobilization and leaching (Salt *et al.* 1995; Pilon-Smits & Pilon 2002). Phytostabilization, analogous to the conventional capping as described below, contains the HM on site without the need for a physical barrier, while contributing to ecosystem integrity. Conversely, phytoextraction via accumulation in the harvestable plant tissues provides a more efficient method of remediating HM polluted media than conventional excavation methods. Not only do the plant tissues accumulate a higher concentration of HM by mass, but they can also be further processed *in situ* (e.g. via chipping) for transportation or storage, or more interestingly, *ex situ* (e.g. via incineration) in order to recover the HM for safe storage or recycling. This concept has been demonstrated through phytomining activities to extract precious metals, such as gold and nickel, into plant tissues, which can then be incinerated to recover the metals (Abou-Shanab *et al.* 2003). Less common is phytovolatilization, in which some HM, such as Hg, As, and Se, can be taken up by the roots and volatilized, diluting their concentration over a larger area (O'Brien *et al.* 2012; Santoyo *et al.* 2016).

Conventional technologies for the remediation of HM use various physical (e.g., excavation or capping) or chemical (e.g., chelating agents or soil washing) methods to either remove (*ex situ*) or contain (*in situ*) the pollutants (as reviewed in Sessitsch *et al.* 2012). In practice, these methods can be effective and are often selected by regulatory bodies and project managers because they are predictable, broadly applicable, and meet regulatory timelines. However, conventional remediation methods are very expensive and can also be energy intensive, produce secondary pollution, and disrupt habitats (Alqueres *et al.* 2013). Consequently, the scale of the problem and the high cost of traditional engineering-based remediation methods have limited clean-up efforts to only the most contaminated sites, resulting in increased public exposure to carcinogens and a loss of economic productivity for moderately affected sites.

Phytoremediation, which leverages the natural abilities of some plants to tolerate and accumulate HM, is a low-cost, environmentally sustainable technology to expand the reach of conventional remediation efforts. Phytotoxicity and habitat suitability are the essential considerations in plant selection for the phytoremediation of HM, and early research focused on identifying plant species with high tolerance to the various HM (O'Brien *et al.* 2012).

Hyperaccumulators are plant species that have the potential to accumulate HM in their tissues to levels far greater than non-accumulating plants, often at levels several orders of magnitude greater than those of other species growing in the same conditions (Silver & Phung 2005). More than 400 hyperaccumulators have been identified for various HM, including plants from some well-known families, such as Asteraceae, Brassicaceae, Fabaceae, and Poaceae (Li *et al.* 2012). The family Brassicaceae, with the genera *Thlaspi*, *Alyssum*, and *Brassica* contain the most known hyperaccumulator species (Zhu *et al.* 2014). Some hyperaccumulators are specialists, like the arsenic hyperaccumulator, *Pteris vitatta*, which has been shown to accumulate 13,800

PPM (mg kg^{-1}) of As in its fronds. Other plants can accumulate multiple HM to high levels, e.g., the pennycress *Thlaspi praecox* Wulf. (Brassicaceae) accumulates high levels of Cd (5,950 PPM), Pb (3,500 PPM), and Zn (14,590 PPM) in its above ground tissues (Zhu *et al.* 2014). While able to accumulate high levels of HM, hyperaccumulators tend to be slow growing, have low biomass, and narrow habitat ranges, limiting their use in phytoremediation (Rajkumar *et al.* 2013).

More recently, woody plant species, such as poplars and willows, are being recognized as a good alternative to HM hyperaccumulators. While they accumulate lower concentrations of HM in their above-ground tissue than do hyperaccumulators, woody trees compensate for the decreased concentration with high biomass, deep roots, and broad habitat ranges (Shin *et al.* 2012; Wan *et al.* 2012; Zhu *et al.* 2014; Babu *et al.* 2015). For example, in a comparison of data between separate studies, Rosenberg *et al.* (1977) reported that while the hybrid poplar (*P. tremula* × *P. alba*) accumulated a lower concentration of Cd in the shoot tissues than the herbaceous Cd hyper-accumulator *T. caerulescens* (11 mg kg^{-1} vs. 40 mg kg^{-1}, dry biomass), the *Populus* trees extracted more total Cd per plant (24.2 mg vs. 1.2 mg per plant, dry biomass) and per hectare (estimated at 250 g Cd ha^{-1} vs. 125 g Cd ha^{-1}).

Phytoremediation offers a good alternative to conventional remediation technologies, especially in moderately contaminated sites. Improving phytoremediation technology requires reducing phytotoxicity, increasing uptake and translocation of the HM into the harvestable tissues, and improving the rate and efficiency of HM extraction. Accordingly, research has included selective breeding programs, genetic transformation, and harnessing the plant microbiome (Farooq *et al.* 2016). The first two methods, selective breeding and genetic transformation, are subject to long-generation times of plants and expensive regulatory hurdles, respectively. The plant

microbiome, on the other hand, presents a novel and less explored approach to improving phytoremediation outcomes.

Endophytes for enhanced remediation of inorganic pollutants

Most research on beneficial microbe-plant interactions in phytoremediation has focused primarily on the rhizosphere, as it is the soil-plant interface (Paez-Espino *et al.* 2009). Microorganisms in the rhizosphere can assist in the phytoextraction of HM by 1) increasing root-soil surface area through increased root biomass via the synthesis of auxin, 2) increasing solubility and changing the speciation of HM through alteration of soil pH or the production of organic ligands, and/or 3) production of organic acids and siderophores that complex cationic ions or desorb anionic ions. However, as the plant-microbiome constituents most intimately associated with the host plant, endophytes are in a unique position to assist in improving HM phytoremediation outcomes.

Endophytes can assist in phytoremediation through reducing phytotoxicity and stimulating host plant growth and development. Studies indicate that endophytes have the capacity to alleviate metal-induced phytotoxicity and enhance biomass production of plants growing under HM stress in laboratory and greenhouse conditions. The endophyte *Rahnella* sp. JN6 isolated from *Polygonum pubescens* growing in multi-HM contaminated soils showed very high Cd, Pb and Zn tolerance as well as the synthesis of the important plant growth promotors, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, indole-3-acetic acid (IAA), and siderophores (Paez-Espino *et al.* 2009). In subsequent pot experiments, the Cd-hyperaccumulators *Amaranthus hypo-chondriacus*, *A. mangostanus*, and *Solanum nigrum* inoculated with *Rahnella* JN27 isolates showed significant increases in biomass and Cd concentrations in both the roots and shoots as compared to un-inoculated controls (Farooq *et al.* 2016). While the exact mechanisms by which

the endophyte promoted increased tolerance were not identified in this study, it is notable that the endophytes were inoculated in novel (i.e., different from the original) hosts and that tolerance and accumulation of Cd were improved in the novel hosts. Broad host range is common in culturable endophytes and represents an important implication for the design of endophyte-assisted phytoremediation systems, one that allows a pairing of the best endophyte(s) and plant(s) for a given pollutant and habitat/site conditions.

Endophytes can also promote phytoremediation through interactions with the host plant's abiotic stress response. Ethylene, an essential plant hormone, is important for normal plant growth and development, and plays a key role in plant responses to a wide range of biotic and abiotic stresses, including metal stress (Farooq *et al.* 2016). However, an overproduction of ethylene in response to stress can inhibit plant developmental processes, such as root elongation, lateral root growth, and root hair formation, which can be as detrimental to plant health as the actual stressor (Wang *et al.* 2002; Zhu *et al.* 2014; Xu *et al.* 2016; Mukherjee *et al.* 2018). Many endophytic bacteria produce the enzyme 1-aminocyclopropane-1-carboxylase (ACC) deaminase which hydrolyzes ACC, a precursor in the synthesis of ethylene, into α-ketobutyrate and ammonia, which is used by the microbes as a source of nitrogen (Mukherjee *et al.* 2018). Several studies have shown that heavy metal-resistant and ACC deaminase-producing bacteria can enhance metal uptake by plants (Mukherjee *et al.* 2018). Specifically, it was demonstrated that *Brassica napus* inoculated with the high ACC deaminase-producing endophytes, *Ralstonia* sp. J1-22-2, *Pantoea agglomerans* Jp3-3, and *Pseudomonas thivervalensis* Y1-3-9, were found to increase the biomass and above-ground tissue Cu contents when cultivated in Cu-contaminated substrates compared to the uninoculated controls (Flemming & Wingender 2010).

HM exposure can induce severe oxidative stress in plants by disturbing cellular equilibria between the generation and the neutralization of reactive oxygen species (ROS). ROS, such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), are constitutively produced at low levels during normal metabolic processes and at high levels in response to various stresses, including HM and invasion by pathogens (Pal & Paul 2008). Successful colonization of host plants by endophytes requires that they evade the host's defense response, and so it is not surprising that genes for the detoxification of reactive oxygen species are prevalent among endophytes (Harrison *et al.* 2007; More *et al.* 2014; Sharma *et al.* 2019). For example, the endophyte *Gluconacetobacter diazotrophicus* PAL5 showed increased expression of genes encoding for ROS-detoxifying enzymes such as superoxide dismutase and glutathione reductase during the colonization of rice roots (Harrison *et al.* 2007). As with ethylene, the overproduction of ROS species can be detrimental to plant health, and a delicate homeostasis must be maintained (Babu *et al.* 2013). The expression of ROS-detoxifying genes by endophytes may moderate the host plant response to HM stress, improving phytoremediation outcomes.

HM tolerance is widespread in microorganisms, including endophytes, and most exhibit tolerance to one or more heavy metals (Li *et al.* 2012; Rajkumar *et al.* 2009). HM tolerant endophytes belonging to a wide range of taxa have been isolated from both hyperaccumulator and non-hyperaccumulator plants (Li *et al.* 2012). For example, a total of 41 As-tolerant endophyte strains, representing 12 genera, were isolated from the shoot and root tissues of the arsenic hyperaccumulators, *P. vittata* and *P. multifada* (Zhu *et al.* 2014). These isolates showed tolerance to both arsenate (AsV) and arsenite (AsIII), as well as the synthesis of key plant growth promoting products, i.e., indole acetic acid (IAA) and siderophores (Zhu *et al.* 2014). In a survey of non-hyperaccumulators growing in soils moderately contaminated with As, Cd, and Pb, our own

research has identified arsenic tolerant endophytes in nearly every plant family assayed, including Ericaceae, Berberidaceae, Rosaceae, Pinaceae, Sapindaceae (Tournay *et al.*, unpublished).

Endophytic bacteria have several mechanisms for their own resistance and detoxification of HM that may benefit the host plant in phytoremediation. These include efflux of metal ions to the exterior of the cell, transformation of metal ions into less toxic forms, sequestration of metals on the cell surface or in extracellular polymers, and precipitation, adsorption/desorption or biomethylation (Rajkumar *et al.* 2013). Unlike with organic pollutants which can be metabolically degraded, there is much less evidence that endophytes interact directly with the toxic metals to alleviate HM stress. However, some recent studies suggest that endophytic bacteria may alleviate metal phytotoxicity via mechanisms that reduce *in planta* concentrations, e.g., precipitation, adsorption, intracellular accumulation and sequestration, or via biotransformation of the toxic metal ions to less- or non-toxic forms, e.g., chelation to metal-binding ligands (Shin *et al.* 2012; Wan *et al.* 2012; Zhu *et al.* 2014; Babu *et al.* 2013).

Arsenate (AsV), the most common arsenic species in oxidized environments, is a chemical and physical phosphate analog and is taken-up by plants via phosphate transport pathways (Rosenberg *et al.* 1977). Once taken up by plants, AsV is reduced to AsIII via arsenate reductase (ARC) in the roots, and then either extruded out of the roots or complexed with phytochelatins (PCs), where the AsIII-PCs complexes are then sequestered in the vacuoles (Farooq *et al.* 2016). Microbes acquire and detoxify arsenate through a similar pathway, acquisition via phosphate uptake and reduction via arsenate reductase (ArsC) (Paez-Espino *et al.* 2009). However, with most microbes, the majority of the AsIII is rapidly expelled from the cell via an arsenite efflux system (ArsB, ArsAB) (Paez-Espino *et al.* 2009). Interestingly, despite its 100-fold higher toxicity, about 90% of As in plant tissues is found in the form of AsIII, even when plants are exposed to AsV

(Farooq *et al.* 2016). This is likely because AsIII reacts efficiently with the thiol-containing compound, glutathione, and phytochelatins (PCs) (Farooq *et al.* 2016). The microbial arsenate detoxification operon *ars* is widely distributed in endophytes, and the ability of endophytes to reduce AsV to AsIII is well documented (Wang *et al.* 2012; Zhu *et al.* 2014; Xu *et al.* 2016; Mukherjee *et al.* 2018). An As-tolerant endophytic consortium of seven endophytes isolated from *Lantana camara* was found to improve the As-phytoremediation efficiency of *Solanum nigrum* (Mukherjee *et al.* 2018). Notably, it was determined that the endophytic consortium expanded the glutathione pool and increased arsenate reductase activity, and the reduction of AsV to AsIII was increased both in shoots and roots of consortium-treated plants as compared to the un-inoculated controls; 68% and 51%, respectively (Mukherjee *et al.* 2018).

Another potential detoxification mechanism in which endophytes might directly interact with HM in planta is the formation of biofilms. Biofilms are assemblages of microorganisms embedded in a matrix of extracellular polymeric substances (EPS), and they form in response to a wide range of environmental stresses, e.g., shear, desiccation, UV, predation, toxins, and bactericides (Flemming & Wingender 2010). Biofilms consist of cells immobilized in a water saturated EPS matrix that is composed primarily of polysaccharides and proteins, with smaller amounts of nucleic acids, lipids, extracellular DNA (eDNA), and other substances (Pal & Paul 2008). In clinical bacterial strains, biofilms have been associated with increased microbial resistance to both antibiotics and toxic metals, while the biofilms of environmental bacteria isolated from acid-mine drainage have been investigated for their ability to remove metals from industrial waste waters (Harrison *et al.* 2007; More *et al.* 2014; Sharma *et al.* 2019). The complex EPS architecture provides an environment rich in charged functional groups, e.g., carboxylate ($R\text{-}COO^-$), phosphate ($R\text{-}HPO_4$), sulfhydryl (R-SH), aminos ($R\text{-}NH_3^+$) and phenol ($R\text{-}C_6H_4OH$), that

may act as potential binding sites for metal ions (Harrison *et al.* 2007). The HM adsorption capabilities of endophytic biofilms might act as a sequestration or detoxification mechanism by reducing free HM concentrations *in planta*, reducing phytotoxicity and improving phyto-remediation outcomes. Transmission electron microscopy (TEM) used in an in vitro study of the endophytic bacteria *Pseudomonas koreensis* AGB-1, isolated from roots of *Miscanthus sinensis*, cultured in a soil extract containing 10 mg As, 5 mg Cd, 5 mg Cu, 30 mg Pd and 20 mg Zn L^{-1}, revealed HM accumulations in the extracellular matrix and at bacterial cell surfaces (Babu *et al.* 2015). Another *in vitro* study utilizing TEM revealed that Pb accumulated in both the extracellular matrix and within the cells of the endophyte *Pseudomonas marginalis* MN3-4 (Shin *et al.* 2012). The oxyanion arsenate (AsV) may also be sequestered in endophytic biofilms. Our research with the endophyte *Rahnella* sp. PD12R, isolated from *Vaccinium parvifolium* growing on arsenic contaminated soils, produces prodigious amounts of EPS under arsenic stress, and is highly tolerant to both AsV and AsIII, growing at 500 mM and 50 mM respectively (Tournay *et al.*, unpublished). For both AsV and AsIII, EPS synthesis increased with increasing arsenic concentration in the media, and the arsenic preferentially localized in the EPS compared to the cells (Tournay *et al.*, unpublished). While these results are promising, to date there has been no report of endophytic biofilms accumulating HM in planta.

Woody tree species as potential phytoremediation candidates

Although they are not hyperaccumulators, the secondary ecological and economic benefits of woody tree species, e.g., poplars and willows, has made them attractive candidates in the phytoremediation of moderately HM contaminated soils. Over the past several decades, field trials have been conducted at former agricultural, industrial, and mining sites contaminated with Cd, Zn, Pb, As, Ni, and Cu; mainly in Europe, but also in Asia and North America (as reviewed in

Vangronsveld *et al.* 2009; Zalesny *et al.* 2019). Despite these efforts, the use of woody tree species in the phytoremediation of HM contaminated soils is still at the developmental stage, with the most significant disadvantage of this technology being the time required to complete the projects (Vangronsveld *et al.* 2009).

Recently the potential of the plant-microbiome has been recognized improve outcomes in the phytoremediation of HM contaminated soils. Most research in this area involves rhizospheric microbes with the goal of increasing the bioavailability and uptake of HM into the host plant, and as with phytoremediation research in general, most of the focus has been on the hyperaccumulators (Burd *et al.* 2000; De *et al.* 2011; Das *et al.* 2016; Mishra *et al.* 2017). Much less attention has been given the potential role of endophytes to improve tolerance, uptake, and translocation of the HM once they have entered the host plant, particularly in woody tree species.

Conclusions

Environmental pollution is a serious threat that must be addressed. Conventional methods of remediation are proven effective, but their high cost has rendered many contaminated sites untreated, with repercussions to humanity and the environment. Genetic engineering of plants, often with genes from pollutant-degrading bacteria, has been a successful approach for overcoming some of the challenges of phytoremediation. However, current regulations limit their utilization. Phytoremediation partnered with microorganisms adapted to deal with pollutants is now a proven technology that is readily deployable. With widespread adoption, endophyte-assisted phyto-remediation has the potential to restore damaged ecosystems and reduce public exposure to carcinogens, ultimately reducing the incidence of cancer and other pollution-caused diseases.

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Chapter 2

The isolation and characterization of arsenic-tolerant endophytes and the potential role of biofilms in arsenic tolerance

Abstract

The endophytes, *Pseudomonas* PD9R and *Rahnella* PD12R, were isolated from native plants growing on arsenic-contaminated soils, and both strains showed tolerance to arsenate (AsV) and arsenite (AsIII). PD9R grew at concentrations up to 100 mM AsV and 10 mM AsIII, while PD12R grew at the highest arsenic concentrations tested, 500 mM AsV and 50 mM AsIII. It was observed that both strains produced extracellular polymeric substances (EPS) when exposed to arsenic. Biofilms are a common defense mechanism against various biotic and abiotic stresses, including toxic metals. PD9R and PD12R formed biofilms when exposed to arsenic, and in PD12R the quantity of EPS and biofilms increased with increasing arsenic concentrations. Further, PD12R biofilms were found to adsorb arsenic by ~2:1 ratio in the EPS fraction vs. the cellular fraction. Combined, the EPS and cellular fractions reduced the total AsV in the media by 4% in 48 hrs.

The genomes of PD9R and PD12R were sequenced and annotated. PD9R has a single 6.88 Mb chromosome and a 27.3 kb plasmid, while PD12R has a 5.51 Mb chromosome and a small 6.1 kb plasmid. The strains were identified via gANI protocols and found to be novel strains of *Pseudomonas* sp. PD9R and *Rahnella* sp. PD12R. Both strains contain arsenic detoxification genes, with PD9R possessing genes associated with the detoxification of AsV, AsIII, and organoarsenicals, and the genes for ArsJ, the only known arsenate efflux protein. PD12R has genes for the detoxification of AsV and organoarsenicals, but no identified genes for the detoxification of AsIII, despite showing high tolerance. It is hypothesized, therefore, that strain PD12R relies more on biofilm formation as the mechanism for arsenic tolerance.

Introduction

Arsenic is an inorganic pollutant of global concern. A naturally occurring element, arsenic is ubiquitously distributed in soils at ~3-4 parts per million (ppm, mg kg^{-1}), but occasionally occurs at elevated levels as a result of natural phenomena or human activities (Chappell *et al.* 1997). Chronic arsenic exposure is linked to cancers of the skin, lung, and bladder, and is associated with a wide array of non-fatal diseases of the cardiovascular, respiratory, neurologic, and reproductive system. The excess cancer risk associated with lifetime arsenic exposure is approximately 1 in 300, which is 30–300 times higher than other known carcinogens (Naujokas *et al.* 2013). Globally, the World Health Organization (WHO) estimates that more than 200 million people are chronically exposed to elevated arsenic levels through agricultural products and drinking water (Ravenscroft *et al.* 2009; Naujokas *et al.* 2013). In the U.S. arsenic has been identified at 63% of EPA Superfund sites and holds the top position on Agency for Toxic Substances and Disease Registry (ATSDR) National Priority List of Hazardous Substances, which is determined by the frequency of occurrence, toxicity, and risk to the public (EPA 2004, ATSDR 2019).

Historical sources of arsenic pollution are the result of smelting operations to extract arsenic trioxide for use in wood preservatives, household products (e.g., paint, wallpaper), glassware, munitions, agricultural pesticides, and as antimicrobials for livestock feed (ATSDR 2007). While increased awareness of the risks associated with arsenic exposure has reduced production and resulted in the prohibition of arsenic in many products, arsenic is still produced on the global market for use in consumer products, such as gallium-arsenide semiconductors for use in personal electronics, microwaves, and solar cells (ATSDR 2007).

Like the other heavy metals/metalloids (e.g., Cd, Hg, Pb, Sb), the remediation of arsenic presents a unique challenge due to its persistence in the environment and toxicity at low

concentrations. Unlike carbon-based organic pollutants, arsenic cannot be degraded, and therefore, conventional remediation solutions commonly use various physical or chemical techniques to either contain the pollutant, e.g., capping, or remove it from the site, e.g., excavation or soil washing (He *et al.* 2020). However, the scope of the global inventory of polluted soils, which is estimated to be more than 5 million sites covering 500 million hectares, and the high cost of conventional remediation techniques has limited clean-up efforts, leaving large areas of contaminated soils untreated (Li *et al.* 2019).

Phytoremediation is an environmentally sustainable and low-cost remediation technology that could be used to expand the reach of conventional remediation for arsenic contaminated soils, decreasing the risk of public exposure and restoring economic productivity to the affected lands. Phytoremediation leverages the ability of some plants to stabilize, degrade, or accumulate pollutants (Pilon-Smits 2005). It provides an alternative to conventional remediation technologies, which while they can be effective, are expensive, energy intensive, create their own pollution (e.g., dust, noise, appearance), and may require additional chemical inputs (Conesa *et al.* 2012a). Conversely, phytoremediation offers several advantages over conventional remediation, as it is less expensive, requires lower energy and chemical inputs, enjoys strong public acceptance, and provides a range of ecosystem services, e.g., wildlife habitat, soil stabilization, carbon sequestration, and a potential for secondary market products (Conesa *et al.* 2012b; Weir and Doty 2016; He *et al.* 2020).

Plant selection is an important consideration in the phytoremediation of arsenic. The ideal plant species would possess the following traits: (i) high tolerance to the toxic effects of arsenic; (ii) high uptake, translocation, and accumulation of the arsenic in the harvestable tissues; (iii) rapid growth with high biomass production; (iv) deeply rooted with extensive underground coverage;

(v) tolerant to a wide range of habitat conditions, both abiotic and biotic (Pilon-Smits 2005; Dickinson *et al.* 2009). A few plants, like several members of the fern genera *Pteris*, including the well-studied Chinese brake fern, *P. vittata*, are hyperaccumulators of arsenic, able to accumulate arsenic at levels lethal to most other plants ((Tu *et al.* 2002; Kumar *et al.* 2015). Not only are these plants highly tolerant to arsenic, but they also translocate and accumulate high concentrations in their harvestable tissues, facilitating the phytoremediation process. However, the ecological trade-off for high tolerance is that hyperaccumulators tend to be slow growing, with low biomass, shallow roots, and narrow habitat ranges; limiting their use in phytoremediation (Ali *et al.* 2013). Conversely, woody plant species, such as poplars and willows, are being recognized as a potential alternative to hyperaccumulators. While they accumulate arsenic at lower concentrations in their above-ground tissue than do hyperaccumulators, fast growing woody trees compensate with high biomass, deep, broadly distributed root systems, and broad habitat ranges (van Slycken *et al.* 2013; Gómez *et al.* 2019; Hauptvogl *et al.* 2020).

Regardless of the plant strategy, arsenic-hyperaccumulators or arsenic-tolerant species, there lies several challenges to improving phytoremediation outcomes, such as reducing phytotoxicity, and increasing the uptake and translocation into the harvestable tissues. Further, as compared to conventional remediation technologies, phytoremediation is much slower and less predictable, two important considerations for commercial remediation contractors, whose profitability is dependent on completing remediation projects under strict project timelines while meeting quality control standards (Conesa *et al.* 2012b). Accordingly, phytoremediation research has focused on addressing these challenges via selective breeding programs, genetic transformation, and harnessing the plant microbiome. The first two methods, selective breeding and genetic transformation, are subject to long-generation times of plants and expensive regulatory

hurdles, respectively. The plant microbiome, on the other hand, presents a relatively new approach to improving phytoremediation outcomes.

The plant microbiome is composed of the microorganisms that colonize the interior and exterior surfaces of plant tissues. While much attention is paid to the relatively small proportion of the plant microbiome that is pathogenic, plants also host mutualistic microbes whose activities directly and indirectly benefit their host (Glick 1995; Ryan *et al.* 2008; Kandel *et al.* 2017). These beneficial microorganisms play an important role in tolerance to abiotic and biotic stresses and can improve host plant fitness under challenging conditions. Members of the plant microbiome improve their host plant's tolerance to stress through various metabolic mechanisms termed plant growth promoting (PGP) factors. These include the production of phytohormones, antibiotics, antioxidants, facilitation of nutrient acquisition, and suppression of induced stress responses (Glick 1995; Reinhold-Hurek and Hurek 2011; Rho *et al.* 2018).

As the plant-microbiome constituents most intimately associated with the host plant, endophytes are in a unique position to assist in improving phytoremediation outcomes. Endophytes are microorganisms that colonize the internal tissues of plants for all or part of their lifecycle, and include bacteria, archaea, and fungi (Hardoim *et al.* 2015). In exchange for photosynthates and reduced competition, endophytes stimulate host plant growth through nutrient acquisition, e.g., phosphate solubilization (Varga *et al.* 2020), nitrogen fixation (Knoth *et al.* 2014), siderophore production (Rashid *et al.* 2012; Tiwari *et al.* 2016); the production of phytohormones, e.g., indole-3-acetic acid (IAA), cytokinin, and gibberellin (Taghavi *et al.* 2009; Gu *et al.* 2018); through modulation of ethylene stress response via the synthesis of ACC-deaminase (Zhang *et al.* 2011; Zhu *et al.* 2014); or the suppression of pathogens via competition and antibiotic production

(Taghavi *et al.* 2010). These traits could *indirectly* improve outcomes in the phytoremediation of arsenic through reduced phytotoxicity and enhanced biomass production.

There is potential for arsenic-tolerant endophytes to *directly* improve phytoremediation outcomes, such as by interacting with the arsenic *in planta* to reduce toxicity or availability to the host plant. This has been demonstrated with organic pollutants, e.g., toluene and trichloroethylene ((Taghavi *et al.* 2005; Doty *et al.* 2017), which can be mineralized by some plants and microorganisms. The microbial genes for arsenic have been well characterized and consist of different strategies for detoxifying arsenic, including cytoplasmic arsenate reduction, arsenite oxidation, and methylation (Silver and Phung 2005; Páez-Espino *et al.* 2009; Yang and Rosen 2016). The most common arsenic detoxification system in bacteria is the *ars* operon. It appears in a variety of genomic configurations, can be chromosomal and/or plasmid-encoded, and the core genes of the system include the transcriptional repressor *arsR*, the arsenite efflux pump *arsB*, and the arsenate reductase *arsC* (Silver and Phung 2005). Some versions of this operon may also include additional genes, e.g., *arsA*, an ATPase that provides energy to ArsB for the extrusion of arsenite, the dual function *arsD*, which is both an arsenite chaperone for ArsB and regulator that controls the upper-level expression of the *ars* operon, or *arsH*, which confers tolerance to trivalent organoarsenicals (Páez-Espino *et al.* 2009).

The reduction of AsV to AsIII via endophytic arsenate reductase could be a potential direct mechanism in promoting host plant tolerance to arsenic. At first glance this seems contradictive given that AsIII is 100x more toxic than AsV (Farooq *et al.* 2016). However, the reduction of AsV to AsIII is part of the plant arsenic detoxification pathway. AsV, the most common inorganic arsenic species in oxygenated environments and the dominant form in aerobic soils, is a chemical and physical analog of phosphate. It is taken up by plants via phosphate transport pathways

(Rosenberg *et al.* 1977), where it is rapidly reduced to AsIII via arsenate reductases (ARC) in the roots, and then either extruded out of the roots, or complexed with glutathione based phytochelatins (PCs) for transport and sequestration in the vacuoles (Farooq *et al.* 2016). An As-tolerant endophytic consortium of seven endophytes was found to improve the As-phytoremediation efficiency of *Solanum nigrum*, which the authors attributed to an expanded glutathione pool and increased arsenate reductase activity (Mukherjee *et al.* 2018).

Endophytic biofilms could be another direct detoxification mechanism in which endophytes might reduce "free" arsenic concentrations *in planta*. The formation of biofilms, communities of microorganisms embedded in a water saturated matrix of extracellular polymeric substances (EPS), are a common response to abiotic and biotic stresses (Flemming and Wingender 2010). Biofilm formation is a common trait in endophytes and has been shown to be required for successful colonization of the host plant in some systems (Liu *et al.* 2017). Biofilms on and within plants have been visualized through the use of fluorescent protein labeling (*fp*) enabling visualization of the localization and aggregation of cells *in planta* (Kandel *et al.* 2017). In clinical bacterial strains, biofilms have been associated with increased microbial resistance to both antibiotics and toxic metals, while the biofilms of environmental bacteria isolated from acid-mine drainage have been investigated for their ability to remove metals from industrial waste waters (Harrison *et al.* 2007; More *et al.* 2014; Sharma *et al.* 2019). A few recent i*n vitro* studies have demonstrated HM accumulations in endophytic biofilms, localized within the EPS matrix and cells (Shin *et al.* 2012; Babu *et al.* 2015).

The main purposes of this research were to (1) extract and isolate As-tolerant endophytes from plants growing in As-contaminated soils; (2) screen the As-tolerant endophytes for the ability to colonize a novel host and improve tolerance to As in that host; (3) investigate endophytic

biofilms as a potential direct mechanism for detoxification of As; and (4) sequence, assemble, and annotate the whole genomes of the As-tolerant endophytes PD9R and PD12R for the purpose of identifying the genes associated with arsenic and biofilm formation. The outcomes of this research will contribute to the understanding of how beneficial endophytes might improve phyto-remediation outcomes for arsenic contaminated soils.

Methods

Endophyte extraction and isolation. The endophytes were isolated from native plants growing on undisturbed forest soils near Pt. Defiance Park, Tacoma, Pierce County, WA (47°18'57.3" N, 122°31'41.0" W) and Maury Island Marine Park, Vashon, King County, WA (47°22'44.8" N, 122°24'19.8" W). Both sites are located within the ASARCO smelter plume (Ecology 2004). The roots and shoots from a total of 38 plants were collected, rinsed with sterile deionized water, and stored at 4 °C until processed (Table S1).

A total of 72 plant tissue samples were processed, with the roots and shoots handled separately. Plant cuttings were surface sterilized with consecutive washes of 10% bleach (10 min), 1% iodophor (5 min), and 75% ethanol (1 min), and rinsed 3x with sterile deionized water after each step (Doty *et al.* 2009). A 100 µL aliquot of the final rinse was plated on MG/L agar and incubated for 24 hours to verify surface sterilization. The sterilized cuttings were trimmed, macerated in 10 mL of MS broth, and 100 µL aliquots of the plant extracts were plated on MS 500 µM AsV agar plates and incubated for 24-72 hours.

Standard media and culturing methods. The following media and growth conditions were used unless noted otherwise. Murashige and Skoog (MS, MM519, Sigma-Aldrich, (Murashige and Skoog 1962)) was used as the primary growth and experiment media because the

nutrient and mineral levels better represent *in planta* conditions than traditional microbiology media. The MS broth was supplemented with 3% glucose, 0.5% mannitol, 15 mM HEPES (pH 6.75), and for plates, 1.5% Bactoagar (BD Difco, Fisher Scientific). Arsenate (AsV as sodium arsenate dibasic heptahydrate, Na_2HAsO_4 $7H_2O$, Fisher Scientific, CAS 10048-95-0) was the primary arsenic species used because it is the dominant arsenic species in oxidizing conditions (Oremland and Stolz 2003). Where noted, arsenite was used (AsIII as sodium arsenate, $NaAsO_2$, Fisher Scientific, CAS 7784-46-5). The strains were grown in MS broth with 2 mM arsenate to maintain arsenic tolerance.

For experiments, cells from -80 °C glycerol stocks were cultured overnight on Mannitol Glutamate/Luria-Bertani (MG/L, (Cangelosi *et al.* 1991)) 1.5% agar plates. Single colonies were enriched in 10 mL MS 2 mM AsV broth for 48 hours on the orbital shaker (150 RPM, 30 °C), washed 3x in phosphate buffered saline (PBS, pH 7.4), resuspended in PBS, and the final OD_{600} recorded. For experiments, suspensions were diluted to a starting OD_{600} of 0.1, incubations were done at 30 °C for solid media, and 30 °C and 150 RPM for liquid media.

For the arsenic tolerance experiments, the optical densities (OD) were recorded using either a bench top spectrophotometer (Fischer Scientific) at OD_{600}, or a 96-well plate reader (Thermo Scientific, Multiskan FC) at OD_{570}. For the biofilm formation experiments the OD were recorded on the 96-well plate reader at OD_{540} (Merritt *et al.* 2005).

Inoculation of a novel host, *Arabidopsis thaliana*. *A. thaliana* seeds (ecotype Col-0) were surface sterilized in a solution of 50% bleach and 0.5% Tween® for 20 minutes, and then stratified at 4 °C for 3 days in sterilized water (Huala *et al.* 2001). Three aliquots of *A. thaliana* seeds (5 mg, dry wt., ~250 seeds) were placed into sterile petri dishes (60 mm) and inoculated with 1 mL inoculum of either PD9R, PD12R, or MS (control). After 24 hours, individual seeds were

transferred using sterile pipette tips onto the surface of MS agar plates supplemented with either 50 µM AsV, 100 µM AsV, or MS control (0 AsV), creating a two-factor experimental design of arsenate (± AsV) and endophytes (± E), with ten seeds per plate and four plates per condition (n = 4). The lids were secured with Parafilm® and the plates were placed on edge in a growth chamber (16 hr. photoperiod, 150 µmol/m^2, 23 °C). At 7 days, the root lengths were measured from digital photographs using ImageJ software (Schneider *et al.* 2012). To estimate the root lengths, each plate was photographed with a ruler in frame. The scale was set for each photograph and the sum of the root lengths of all plants were measured using the ImageJ *Line Selection Tool*. At 14 days, the plants were destructively harvested, dried for 24 hr. at 80 °C, and the total biomass (mg, dry wt.) per plate was recorded.

Optical density measurements during growth in arsenic. To estimate cell growth in arsenic, OD_{600} readings were recorded for cell suspensions of PD9R and PD12R over a 48-hour period. The cell suspensions, 10 mL in 125 mL Erlenmeyer flasks, were completed in triplicate (n = 3) and included an MS control (0 AsV). PD9R was tested at AsV concentrations of 100 µM, 500 µM, and 2 mM, and PD12R at AsV concentrations of 250 µM and 1 mM. The OD_{600} readings of 1 mL aliquots (diluted 1:10 as necessary) were recorded at 6, 12, 24, 36, and 48 hours.

A follow-up experiment was conducted on both strains with AsV concentrations ranging from 10 mM - 80 mM plus an MS control (0 AsV), with 3 replicates per strain and AsV concentration (n = 3). For each strain, 100 µL aliquots were incubated in 96-well plates, and the OD_{570} values were recorded at 24 and 36 hours.

Colony-forming units (CFU). CFU were then used to estimate viable cells for PD9R and PD12R grown at AsV concentrations ranging from10 mM - 80 mM plus MS control (0 AsV). For each strain, 2 mL cells suspensions were cultured in 12-well plates (Corning, 3513, Fisher

Scientific) with 9 replicates per AsV concentration (n = 9). At 24 and 36 hours, 100 µL aliquots of serial dilutions (10^{-4} to 10^{-6}) were plated on MG/L agar and CFU counted after incubation for 24 hrs.

Determination of the minimum bactericidal concentration (MBC) of PD9R and PD12R. To estimate the minimum bactericidal concentration (MBC) of arsenic (Harrison *et al.* 2007) for PD9R and PD12R, 100 µL aliquots in 96-well plates cells were cultured in AsV concentrations ranging from 25 mM – 500 mM and AsIII concentrations ranging from 1 mM – 20 mM (n = 3). At 24 and 36 hours, 5 µL aliquots were plated on MG/L agar and the presence or absence of growth recorded after incubation for 24 hrs.

Biofilm formation under arsenic stress. To estimate PD9R and PD12R biofilm formation in the presence of arsenic, 0.1% crystal violet (CV) was used to stain the biofilms of PD9R and PD12R grown in the presence of AsV (1 mM – 80 mM) and AsIII (0.5 mM – 50 mM). The CV-stained biofilms were solubilized in 30% acetic acid and the OD_{540} recorded (Merritt *et al.* 2005). There was a total of 8 replicates for each concentration of AsV and AsIII, respectively, and the experiment was repeated 3 times (n = 24). Briefly, 100 µL cell suspensions were incubated in 96-well plates for 48 hours at 30 °C under static conditions. After incubation, the planktonic cells were discarded, the plates gently washed in water (2x) to remove unattached cells and allowed to air dry for 24 hours. The biofilms were stained with 125 µL of 0.1% CV solution for 15 minutes, and the plates gently rinsed (3x) in water. After 24 hours the stained biofilms were solubilized with 125 µL of 30% acetic acid and the OD_{540} was recorded.

Characterization of the PD12R biofilm. The three previous experiments were repeated on PD12R, estimating the OD_{570}, CFU, biofilm formation of PD12R in a single experiment. PD12R was tested at AsV concentrations from 10 mM - 80 mM plus MS control (0 AsV). For

each concentration, twelve 100 µL aliquots were placed in a 96-well plate, and the experiment was run in triplicate (n = 36). At 24 and 36 hours, the OD_{570} readings were recorded, and then three wells from each treatment were randomly selected, the samples serially diluted ($10^{-5} – 10^{-7}$), plated on MG/L, and after 24 hrs. incubation the CFU counts recorded. At the termination of the experiment (36 hrs.), the biofilms were estimated per the CV staining protocol previously described.

Arsenic adsorption and localization in PD12R biofilms. The biofilm and EPS, defined as the loosely bound soluble (LB-EPS), the tightly bound capsular (TB-EPS) and cell fractions, produced by PD9R and PD12R were estimated under arsenic stress. Sterile polycarbonate membranes (Whatman, pore 0.22 µM, 19 mm) placed on the surface of MS agar supplemented with AsV were inoculated with 15 µL of either PD12R or PD9R and grown for 72 hours at 30 °C (Photo 1) (Comte *et al.* 2006). The AsV concentrations were 500 µM, 2 mM, and 5 mM, plus MS controls (0 AsV), with six disks per plate and three plates per treatment (n = 3). The disks and biofilms were removed from the plates and weighed (mg, wet wt.), then subjected to a heated, acid digest and the arsenic concentration (ppm) estimated using Inductively Coupled Plasma Optical Emission spectroscopy (ICP-OES, Thermo Fisher Scientific, Model 6300, SEFS Soil Analytics Lab, University of Washington) per EPA method 3050B. To account for the mass and arsenic absorbed by the membranes, un-inoculated mock plates (n = 3) were included in the experiment and handled identically to the inoculated plates. The mass and AsV concentration of the inoculated disks were reduced by the mean mass and mean AsV concentration of the un-inoculated disks.

To estimate where arsenic localizes in PD12R biofilms, a second experiment was conducted where the cells were grown in 100 mL MS broth supplemented with 2mM AsV for 48 hours, and then separated into the LB-EPS, the TB-EPS, and cell fractions using physiochemical

methods (Comte *et al.* 2006). Briefly, the LB-EPS fraction was extracted as the supernatant after centrifugation for 20 minutes at 10,000 RPM. The remaining pellets were resuspended in sterile deionized water, and then incubated with 5 mL EDTA (pH 8.0) for 3 hours at 4 °C. The TB-EPS fraction was extracted as the supernatant after centrifugation for 20 minutes at 10,000 RPM. The remaining pellets, the cell fraction, were resuspended in sterile deionized water. The three fractions were lyophilized at -50 °C for 48 hours, the masses recorded, and digested for ICP-OES analysis (EPS 3050B). For the LB-EPS and TB-EPS fractions, 0.25 g samples were digested, and the entire mass of the cell fraction was digested.

WGS sequencing, assembly, and annotation of PD9R and PD12R. A standard shotgun library was constructed using Nextera DNA Flex (Illumina, Cat. No. 20018705) and sequenced at University of Washington Northwest Genomics Center (NWGC) on an Illumina MiSeq platform (300-Cycle V2 Reagent Kit). De novo assembly was performed on the resulting 150-bp MiSeq paired-end reads, after quality trimming with the BBDuk plug-in (Version 38.84), in the Geneious Prime assembler in the Geneious Prime platform (v2020.0.3, https://www.geneious.com). Consensus files containing chromosomal contigs greater than 1000 bp and putative plasmid contigs were annotated separately using the Random Annotation using Subsystem Technology (RAST) online platform and the genes of interest analyzed using Geneious Prime (Aziz *et al.* 2008). Reference biofilm pathways were examined using the KEGG Automatic Annotation Server (KAAS, https//www.genome.jp/tools.kaas/).

Identification of PD9R and PD12R via 16S rRNA and genome-wide Average Nucleotide Identity (gANI). Colony PCR was performed using Epicenter PCR Pre-Mix buffer B and Taq polymerase (Promega; Madison WI) in an Eppendorf thermocycler, using universal 16S rRNA primers 8F (5'- AGAGTTTGATCCTGGCTCAG-3') and 1492 (5'-

GGTTACCTTGTTAC GACTT-3') as described previously (Doty *et al.* 2005). The thermocycler program was 94°C for 15 s, 44°C for 15 s, 72°C for 30 s and it was repeated for 24 cycles. The 1.5 Kb PCR products were purified using ExoSAP-IT PCR clean-up kit (Thermo-Fisher; Waltham, MA), and sequenced by GENEWIZ (South Plainfield, NJ). The 16S rRNA sequences were subjected to BLASTn alignments in the NCBI database to identify the strains to the genus level (Altschul *et al.* 1997; Tamura *et al.* 2013). The 16S rRNA phylogenetic trees for PD9R and PD12R were constructed by the Neighbor-Joining algorithm using the Tamura-Nei genetic distance model in Geneious Prime (v2021.1.1). Species identification was performed by calculating the genome-wide Average Nucleotide Identify (gANI) and Alignment Fraction (AF) via pairwise alignment of each strain's coding sequences against whole genome CDS from the NCBI/RefSeq database (Varghese *et al.* 2015; O'Leary *et al.* 2016).

Statistics. Statistical tests were completed in R version 4.0.2 (R Core Team 2013), where differences in means were analyzed using either one-way or two-way Analysis of Variance (ANOVA), and when appropriate Tukey's HSD post hoc.

Results

Isolation of arsenic-tolerant endophytes. A total of 72 plant extracts from surface sterilized roots and shoots were screened for arsenic-tolerant endophytes on MS agar with 0.5 mM AsV. Colonies formed on all of the MS 500 µM AsV plates within 72 hours, indicating that a basic level of As-tolerance is common in the endophytes of plants growing on As-contaminated soils. Based on growth at 24 hours, a library of 30 As-tolerant endophytes was constructed by selecting morphologically distinct colonies to be streak-purified 3x on MS 500 µM AsV plates. The As-tolerant endophytes were then screened on MS plates supplemented with either 1 mM, 2 mM, or 4 mM AsV. Of these endophyte strains, 23 strains grew at 1 mM AsV, while 14 strains

grew at 4 mM AsV (Table S1). Finally, 1 mL glycerol stocks of the 23 isolates (66% cell suspension /33% glycerol, v/v) were flash frozen and stored at -80 °C for stocks (Khan and Doty 2009). Two strains, *Pseudomonas* sp. PD9R and *Rahnella* sp. PD12R, were selected for further study.

Inoculation of a novel host, *A. thaliana*. As expected, AsV had a negative effect on both root length and biomass (Table 1). The mean root lengths of the endophyte inoculated plants were greater than the uninoculated plants for both strains, however the difference was only significant in the PD9R inoculated plants ($F_{1,18}$ = 7.60, p = 0.012). The effect on root length appears independent of whether or not the plants were exposed to AsV, and no interaction between AsV and inoculation was observed for neither PD9R ($F_{2,}$ = 2.08, p = 0.153) nor PD12R ($F_{2,18}$ = 0.28, p = 0.759). The response of inoculation on plant biomass was somewhat different, where the inoculated plants had a higher mean biomass only when AsV was in the media. However, the PD12R inoculated plants did not benefit when under arsenic stress ($F_{2,18}$ = 0.15, p = 0.861). Conversely, PD9R did have a positive effect on biomass when AsV was in the media (Figure 1, $F_{2,18}$ = 4.578, p = 0.024), and the mean biomass of the inoculated plants was greater than the uninoculated plants at both AsV concentrations (Tukey's HSD, $F_{1,6}$ = 11.68, p = 0.014 and $F_{1,6}$ = 39.48, p < 0.001, respectively).

Optical density measurements during growth in arsenic. Contrary to expectations, the OD_{600} readings for both strains increased as AsV concentration increased in the media (Figure 2), suggesting that the strains were growing better under arsenic stress ($F_{2,6}$ = 1536.0, p < 0.001 and $F_{2,6}$ = 38.08, p < 0.001, for PD9R and PD12R, respectively). For example, after 36 hours the mean OD_{600} of the PD9R cells in 2 mM AsV was 0.81 ± 0.010, which was more than 4x that of the controls grown in the absence of AsV (0.19 ± 0.016). The pattern was consistent between AsV

concentrations, such that the OD_{600} of the controls ~ 0.1 mM AsV < 0.5 mM AsV < 2 mM AsV (Tukey's HSD, $p < 0.05$). Similarly, at 48 hrs. the OD_{600} of the PD12R cells grown in 1 mM AsV was more than double that of the controls (0.87 ± 0.044 vs. 0.40 ± 0.056), such that the no AsV controls < 0.25 mM AsV < 1 mM AsV ($p < 0.05$).

The follow-up experiment at much higher AsV concentrations (0 AsV – 80 AsV) returned similar results (Figure 2), and the mean OD_{570} values were greater in the cells grown in AsV than when no AsV was in the media ($F_{6,14} = 11.98$, $p < 0.001$ and $F_{6,14} = 4.28$, $p = 0.012$, for PD9R and PD12R, respectively). For both strains, the OD_{570} readings were consistently higher for the cells exposed to AsV than those of the controls, while between the AsV treatment groups the OD_{570} readings tended to be more similar. For example, in PD9R the control group was significantly less than all other AsV treatment groups ($p < 0.05$), while the AsV treatment groups themselves were not significantly different ($p > 0.05$). With the exception of the 80 mM AsV group in the PD12R experiment, the OD_{570} readings for the AsV groups were higher than the control ($p < 0.05$).

Colony Forming Units (CFU). The CFU counts were conducted on both strains to rule out the possibility that the elevated OD reading were caused by cell growth under arsenic stress (Figure 3). While the CFU counts trended down with increasing AsV concentration in the media, no differences in the estimates of viable cells were found for either strain, regardless of whether they were grown in the presence of AsV or not ($F_{2,32} = 1.405$, $p = 0.259$ and $F_{2,32} = 1.755$, $p = 0.176$, PD9R and PD12R respectively).

Minimum bactericidal concentration (MBC). The MBC of PD9R and PD12R were estimated in a range of AsV (25 mM – 500 mM) and AsIII (1 mM – 50 mM), to see the minimum arsenic concentrations at which CFU failed to form. PD9R was more sensitive than PD12R in both

AsV and AsIII, failing to grow above 100 mM AsV and 10 mM AsIII, while PD12R grew at all AsV and AsIII concentrations tested; up to 500 mM and 50 mM, respectively.

Biofilm formation in arsenic. PD9R and PD12R biofilms had very different responses to arsenic stress. PD9R formed biofilms regardless of whether or not arsenic was in the media, but the ability to form biofilms declined as concentrations increased for both arsenic species (Figure 4) ($F_{9,203} = 9.19$, $p < 0.001$ and $F_{9,230} = 72.93$, $p < 0.001$, for AsV and AsIII, respectively). In AsV, the biofilms of the controls (0 AsV) were similar to those formed by the cells exposed to the lower concentrations, but declined at AsV concentrations greater than 10 mM, significantly so at 40 mM and 80 mM AsV ($p < 0.05$). In AsIII, the more toxic arsenic species, PD9R did not reliably form biofilms at AsIII concentrations higher than 6 mM, with more than 50% of the replicates falling below the detectable threshold at 10 mM and 12 mM, and 100% failing at 15 mM and 50 mM AsIII.

In contrast, PD12R formed biofilms at all tested concentrations of AsV and AsIII (Figure 4). The PD12R biofilms increased with the AsV concentration, up to and including 80 mM AsV and 15 mM AsIII ($F_{9,230} = 29.94$, $p < 0.001$ and $F_{9,230} = 20.13$, $p < 0.001$, for AsV and AsIII, respectively). Only at 50 mM AsIII did PD12R experience a reduction in biofilm formation; nevertheless 100% of the replicates formed biofilms at 50 mM AsIII. In AsV, the PD12R biofilms at the lower concentrations, i.e., control (0 AsV) through 5 mM, were similar to each other ($p > 0.05$), but less than those formed at the higher AsV concentrations, i.e., 10 mM through 80 mM. The AsIII biofilms of PD12R followed a similar pattern, with the biofilms of the lower AsIII concentrations, i.e., <= 6 mM, being more similar than those formed at the higher AsIII concentrations, i.e., >=10 mM; with the exception being the biofilms formed in 50 mM AsIII, as noted above.

Characterization of PD12R biofilms. Three previous experiments, growth in AsV, CFU, and biofilm formation, were repeated on PD12R in a single experiment (Figure 5). PD12R was grown in a range of AsV concentrations (0 mM – 80 mM), and the data for optical density (OD_{570}), CFU counts, and biofilm formation (OD_{540}) were collected per previous methods. The patterns observed in this combined experiment were consistent with those of the previous independent experiments, where both optical density (OD_{570}, $F_{4,160} = 176.8$, $p < 0.001$) and biofilm formation (OD_{540}, $F_{4,175} = 94.8$, $p < 0.001$) increased in response to AsV concentration in the media, while CFU did not ($F_{4,10} = 0.2$, $p = 0.921$).

Arsenic adsorption and localization in PD12R biofilms. The mass and AsV ppm of the biofilms, defined here as inclusive of the LB-EPS, the TB-EPS, and the cells (see Photo 1), were estimated under various AsV concentrations. PD9R failed to produce sufficient quantities of EPS in this experiment for analysis and therefore no data have been included. However, PD12R produced a robust biofilm when exposed to arsenic in the media as compared to the no arsenic controls ($F_{3.8} = 44.06$, $p < 0.0001$). Neither the mass nor the AsV concentration was different between the PD12R biofilms formed on the AsV media (Figure 6). While the AsV ppm in the biofilms increased along with AsV concentration in the media, when the results were normalized by the original AsV concentration in the media, there were no differences ($p > 0.05$).

To assess whether AsV associates more closely with the EPS vs. the cell fractions, PD12R cells were cultured in the presence of 2 mM AsV and then separated into their fractions, e.g., the LB-EPS, the TB-EPS, and the cells. The mean mass of the PD12R cells of the controls was more than double (60.27 mg vs. 26.75 mg) that of the cells grown in the presence of AsV ($t = -8.19$, $p = 0.007$) (Figure 7). However, on a per cell mass basis, both the LB-EPS and TB-EPS treatment fractions were greater in the AsV treated cells as compared to the controls ($t = 3.67$, $p = 0.0319$ and

t = 7.12, p = 0.003 respectively). The AsV concentration associated with the LB-EPS could not be distinguished from the AsV in the media, so those results have been omitted. However, the mean AsV ppm in the TB-EPS was 86% greater than the AsV ppm of the cells (t = 4.265, p = 0.007) (Figure 8).

Identification of endophytes PD9R and PD12R. Analysis of 1,400 bp 16S rDNA sequence of strain PD9R using the BLASTn program at NCBI showed > 97.5% similarity to other *Pseudomonas* spp., while the strain PD12R showed > 99.5% similarity to other *Rahnella* spp. (data not shown). The gANI protocol measures genomic relatedness between sequences via two metrics, an average nucleotide identity of orthologous genes (gANI) identified as bi-directional hits (BBHs), and the alignment fraction of those orthologous genes (AF). The authors suggest that species classification thresholds for the gANI and AF scores to be 96.5% and 0.6, respectively (Varghese *et al.* 2015). For *Rahnella* there existed 8 complete genomes published at the time of comparison, while for *Pseudomonas* there were more than 5,000 complete genomes published; dominated by *P. aeruginosa*. First, PD9R was verified to not be a *P. aeruginosa* strain by alignment with a random sample of *P. aeruginosa* genomes (data not shown). Based on those findings, PD9R was compared to a list of ~500 complete genomes from within the *Pseudomonas* genera. None of the published complete genomes exceeded gANI and AF scores for either PD9R or PD12R, suggesting that both strains are novel species (Table 2). For PD9R, the closest match was *P. frederiksbergensis*, gANI (87.23%) and AF (0.74), while the closest match for PD12R was the *Rahnella* sp. ERME1:05, gANI (93.21%) and AF (0.82).

Genome features and relevant annotations of PD9R and PD12R. The *Pseudomonas* sp. PD9R genome consists of a single 6.88 Mb chromosome and a single 27.3 kb plasmid. The chromosome contains a total of 6519 predicted genes, consisting of 6454 protein-coding sequences

and 65 RNAs, with an average GC content of 58.4%. The genome of *Rahnella* sp. PD12R is composed of a single chromosome of 5.51 Mb and a single plasmid of 6.1 kb. A total of 5329 predicted genes were identified, including 5251 protein-coding sequences and 78 RNAs, with an average GC content of 53.3%.

PD9R carries a robust collection of genes for arsenic tolerance and detoxification (Figure 10). The PD9R genome shows two *ars* operons, *ars1* and *ars2*, a single *aioAB* operon, plus an *arsB* gene and three *arsR* genes not associated with operons. The *aioAB* operon encodes for a periplasmic arsenite oxidase than catalyzes the reduction of AsIII to the less soluble AsV under aerobic conditions (Lebrun *et al.* 2003). The two *ars* operons are very different in structure, *ars1* is a four gene operon, *arsRBCH,* and is related to the *arsRBC* operon, which uses thioredoxin as the electron acceptor, and was originally characterized in the *Staphylococcus aureus* pI258 plasmid (Ji and Silver 1992). Conversely, the reversely transcribed *ars2* contains 8 genes, including two arsenate reductases (*arsC*1 and *arsC2*) and an *acr3* gene in place of the *arsB.* As with *ars1*, both *arsC* genes are from the thioredoxin family of arsenate reductases, while the *acr3* gene is related to the arsenite permease originally characterized in *Saccharomyces cerevisiae* (Wysocki *et al.* 1997). Where the *arsB* gene is found only in prokaryotes, the *acr3* gene is found in bacteria, archaea, fungi, and some plants (Fekih *et al.* 2018). The operon also contains an *arsH* gene, which confers tolerance to trivalent organoarsenicals through oxidation of MAs(III), PhAs(III), and Rox(III) to pentavalent forms (Yang and Rosen 2016). Most interestingly, this operon contains the *gapdh-arsJ* gene cluster, which is the only known arsenate efflux protein. The system was only recently characterized in *P. aeurginosa* DK2 (Chen *et al.* 2016). The genes encode an enzyme, GAPDH (glyceraldehyde 3-phosphate dehydrogenase) and a novel MFS transporter, ArsJ. GAPDH catalyzes the formation of an AsV-phosphoglycerate, which is then extruded by the ArsJ

permease. The role of the *dsp* (dual specific protein phosphatase) is unknown in arsenic tolerance, but frequently occurs adjacent to the *gapdh-arsJ* gene cluster (Chen *et al.* 2016).

By comparison, PD12R has a much simpler arsenic tolerance and detoxification system (Figure 10). It contains two similar *ars* operons, *arsRBCH*, and two *arsR* genes not associated with operons, and no identified genes for the detoxification of arsenite. The *ars* operons of PD12R share the same genes, *arsRBCH*, although with slightly different configurations. PD12R *ars1* contains a reverse transcribed *arsH*, an organoarsenical oxidase, immediately upstream from the *arsRBC* cluster. This is an unusual, but not unheard-of configuration (Cile Neyt *et al.* 1997). The PD12R *ars2* operon is composed of the more common *arsRBCH* configuration (Fekih *et al.* 2018).

The PD9R genome contains many of the genes noted in biofilm pathway of the model organism *Pseudomonas aeruginosa*, e.g., polysaccharide biosynthesis (Pel), alginate (Alg), quorum sensing (Lux), cyclic-di-GMP, and oxidative stress (Oxy) (ref. KEGG pathway ko02025, Vasseur *et al.* 2005; Moriya *et al.* 2007; Rasamiravaka *et al.* 2015). Notably, the *psl* genes commonly associated with polysaccharide biosynthesis in *P. aeruginosa* were not identified in the annotated PD9R genome. *Rahnella* biofilms are not so well characterized, but the annotated PD12R genome identified a gene for YceP (BssS), a predicted biofilm regulator protein previously identified in *Escherichia coli* K-12 (Domka *et al.* 2006), in addition to genes for quorum sensing (Lux), cyclic-di-GMP, and oxidative stress (Oxy) (ref. KEGG pathway map02026).

Discussion

Arsenic is a common abiotic stress due its ubiquitous distribution in the environment at low levels, and this has driven the selection and conservation for arsenic detoxification genes in most organisms. Like in plants, tolerance to arsenic varies in microorganisms, with most possessing the ability to tolerate low levels of arsenic, while arsenic "specialists", such as

Sulfurospirillum arsenophilum, are not only tolerant to high arsenic levels, but use arsenate as a terminal electron acceptor during anaerobic metabolism (Oremland and Stolz 2003) Judging by the presence of arsenic detoxification genes alone, it would be a reasonable assumption that the *Pseudomonas* sp. PD9R would be more tolerant to arsenic than the *Rahnella* sp. PD12R. The *Pseudomonas* sp. PD9R hosts a particularly robust arsenal of arsenic detoxification genes, including systems for the detoxification of arsenate, arsenite, and organoarsenicals. The PD12R arsenic detoxification genes are simple by comparison, with only the arsenate and organoarsenical detoxification systems identified, and so it is surprising that PD12R shows a much greater tolerance to arsenic than PD9R.

Preliminary OD_{600} experiments on PD9R and PD12R were conducted in the presence of arsenate in order to test the effects of arsenic stress on the growth of the strains. Unexpectedly, the OD_{600} measurements of both strains increased with increasing AsV concentration in the media. This observation was initially made at low AsV concentrations (> 2 mM AsV), but subsequently confirmed to occur at AsV concentrations as high as 80 mM AsV (Figure 2 & 5). Optical density, commonly used to estimate cell growth in liquid media, is based on the turbidity resulting from light scattering by cells (Koch 1970). While it is an easy and rapid method to monitor the growth of bacteria under experimental conditions, non-viable cells and other suspended substances may reduce the accuracy. The *Rahnella* sp. PD12R strain produces prolific amounts of EPS under arsenic stress (Photo 2), and so we hypothesized that arsenic-induced EPS may have been the cause of the OD readings.

To test that the elevated OD readings were not caused by cell growth in the media, CFU counts were performed on PD9R and PD12R grown in the presence of As and it was found that CFU did not vary between the cells grown in arsenic and the controls (Figure 3). Based on these

findings we decided to investigate whether PD9R and PD12R form biofilms under arsenic stress, and whether they adsorb arsenic. It is well established that bacteria form biofilms as protection from abiotic and biotic stresses, and studies on clinical and environmental bacteria have provided evidence for a protective role for EPS against various bactericidal compounds, including metals and metalloids (Harrison *et al.* 2004; Harrison *et al.* 2007). Increased EPS synthesis in response to various HM has been recorded in bacteria resistant to Cu, Cr, Cd, Zn, Pb, and Hg (Teitzel and Parsek 2003; Kopycińska *et al.* 2018; Leonel *et al.* 2019). The arsenite oxidizing *Herminiimonas arsenicoxydans*, an isolate obtained from industrial sludge, increases EPS synthesis in the presence of arsenic, and *H. arsenicoxydans* biofilms have been shown to trap AsIII ions (Weiss *et al.* 2009; Marchal *et al.* 2010).

Bacterial EPS composition varies widely between species, and also within species according to environmental conditions, although generally it consists primarily of carbohydrates (75 – 90%), with lesser quantities of proteins, lipids, nucleic acids, and substances acquired from the local environment (Costerton *et al.* 1995; Flemming and Wingender 2010b). Depending on the nature of its association with the cell, EPS has been defined as soluble (LB-EPS) or insoluble (TB-EPS), where LB-EPS consists of polymers secreted or lysed from the cells and dissolved into the surrounding environment, and TB-EPS is bound to the cell membrane (Comte *et al.* 2006b; More *et al.* 2014). Accordingly, the elevated OD readings in the previous experiments were likely caused by the LB-EPS secreted into the media by the cells. Conversely, biofilms are described as aggregates of cells attached to surfaces or each other and embedded in an EPS matrix (Flemming *et al.* 2016). To test whether arsenic also induced biofilm formation in the endophytes, we screened PD9R and PD12R in 96-well plates under a range of AsV (0 – 80 mM) and AsIII (0 – 50 mM) (Figure 4).

The biofilm formation of both PD9R and PD12R varied with arsenic concentration in the media, but the responses were very different, with PD9R biofilms decreasing above 10 mM AsV and 6 mM AsIII, respectively. In fact, PD9R failed to form biofilms above 12 mM AsIII. Conversely, the biofilms of PD12R increased at all concentrations of AsV, and all but 50 mM AsIII (Figure 4). The differential response in biofilm formation under arsenic stress was not surprising given the high variability of biofilm formation and EPS synthesis noted in the literature between different bacterial species, and even between closely related strains. A study on the biofilm formation on three arsenic resistant *Thiomonas* strains, CB1, CB2, and CB3, found changes in the architecture and kinetics of the biofilms formed under arsenic stress, but that the response varied from one strain to the other (Farasin *et al.* 2017). While two of the strains favored biofilm formation, the third strain favored motility in the presence of arsenite (Farasin *et al.* 2017) Variability in biofilm formation has been observed in *Pseudomonas* strains exposed to Zn and Pb (Meliani and Bensoltane 2016), *Klebsiella pneumoniae* exposed to Hg (de Araújo *et al.* 2019), and *Sinorhizobium meliloti* strains exposed to Hg and As (Nocelli *et al.* 2016). Prokaryote tolerance to HM is often orders of magnitude greater than in eukaryotes, and so it is worth noting that in many cases the concentrations of HM used in biofilm and EPS studies (including this one) far exceeded the concentrations that would be encountered by plants at even the most polluted sites. Therefore, the effect of high concentrations of HM on endophytic biofilms, such as was observed with PD9R and arsenic, might be less important to phytoremediation outcomes than their ability to adsorb the metals within the biofilms themselves.

Accordingly, we decided to assess the ability of PD9R and PD12R biofilms to adsorb AsV and to estimate the AsV concentration in the biofilms. To do this PD9R and PD12R biofilms were grown on the surface of membranes placed on MS agar with AsV (0 mM – 5 mM). This allowed

for the collection and analysis of the biofilms inclusive of the LB-EPS, TB-EPS, and cell fractions. PD9R failed to grow sufficient mass for ICP-OES analysis and so were excluded from the final analysis. As observed in previous experiments, the masses of the PD12R biofilms exposed to arsenic were greater than the controls (0 AsV). Interestingly, the concentration of AsV (ppm) for all of the PD12R biofilms was ~6.4x the original concentration of AsV in the media (Figure 6), suggesting that PD12R biofilms were not saturated at the experimental AsV concentrations.

In a follow up experiment, PD12R cells cultured in MS media with 2 mM AsV were separated into the LP-EPS, the TB-EPS, and the cell fractions via physiochemical methods and analyzed for AsV concentration to determine where AsV localized in PD12R biofilms. While the AsV associated with the LB-EPS fraction could not be distinguished from the AsV in the media, it was found that the AsV associated with the TB-EPS fraction was estimated to be nearly double that of the cells, 254 ppm vs. 473 ppm. By comparison, a study of the soil bacteria, *Bacillus subtilis* and *Paenibacillus macerans*, grown in 0.67 mM AsIII, found that arsenic adsorption occurred in the EPS by a 12:1 (39.8 ppm vs. 3.32 ppm) and 5:1 (24.37 ppm vs. 4.93 ppm) ratio over the cellular fraction (Vishnoi *et al.* 2014). The soil bacteria *Exiguobacterium profundum* PT2 biofilms adsorbed a mean of 294 ppm AsV after 72 hrs. incubation in media with 10 mM AsV, although this study did not differentiate by cell and EPS fractions (Saba *et al.* 2018).

Conclusions

This study demonstrated that the endophytes *Pseudomonas* PD9R and *Rahnella* PD12R possess tolerance to both AsV and AsIII, and that arsenic induces EPS synthesis and biofilm formation in these strains. PD12R biofilms have some ability to adsorb arsenic, and that it preferentially localizes in the EPS vs. the cells. Those results are supported by the identification of arsenic detoxification and biofilm genes in both strains after sequencing and annotations of the

genomes. Interestingly, the robust arsenic detoxification systems of PD9R did not provide a greater tolerance to AsV or AsIII than the comparatively simple arsenic detoxification mechanisms identified in PD12R. Considering the outcomes of the EPS and biofilm studies suggests that the superior EPS synthesis and biofilm formation by PD12R may complement the arsenic detoxification mechanisms, improving its tolerance to both AsV and AsIII. The continued characterization of the endophytes and their biofilms under arsenic stress will be assisted by the recent availability of the annotated genomes.

Biofilms are transient by nature and so in addition to measuring adsorptive capacity, it would be interesting to explore how arsenic influences endophytic biofilms over time. While the internal environment in plants is less heterogeneous than the external environment (i.e. rhizosphere and soils), the ability of endophytes to form biofilms *in planta*¸ and whether those biofilms adsorb arsenic would be logical future steps. Finally, it was observed in one study on the biofilm forming rhizospheric *Azospirillum brasilense* Cd that siderophore production decreased significantly and the synthesis of IAA declined moderately at under arsenic stress. In this case, it could mean there is a trade-off between a potential benefit of biofilm formation to reduce "free" arsenic levels and a reduction in the PGP benefits to reduce phytotoxic response in the host plant.

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Tables and Figures, Chapter 2

Photos

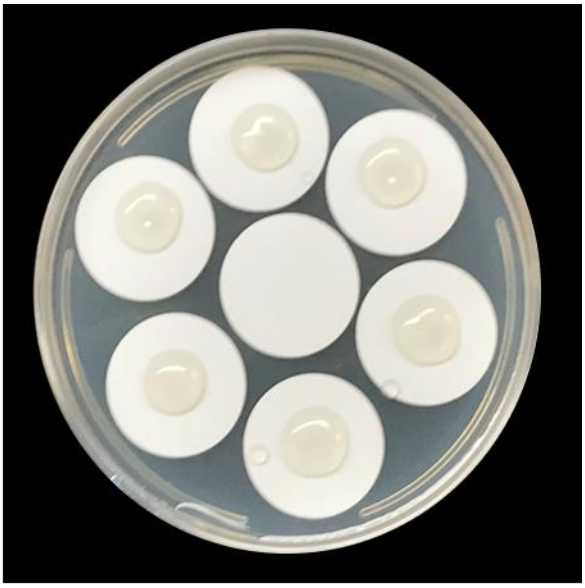

Photo 1. *Rahnella* sp. PD12R biofilms grown on polycarbonate membranes (pore 0.22 µM, 19 mm dia. MS with 2 mM AsV (48 hrs.)

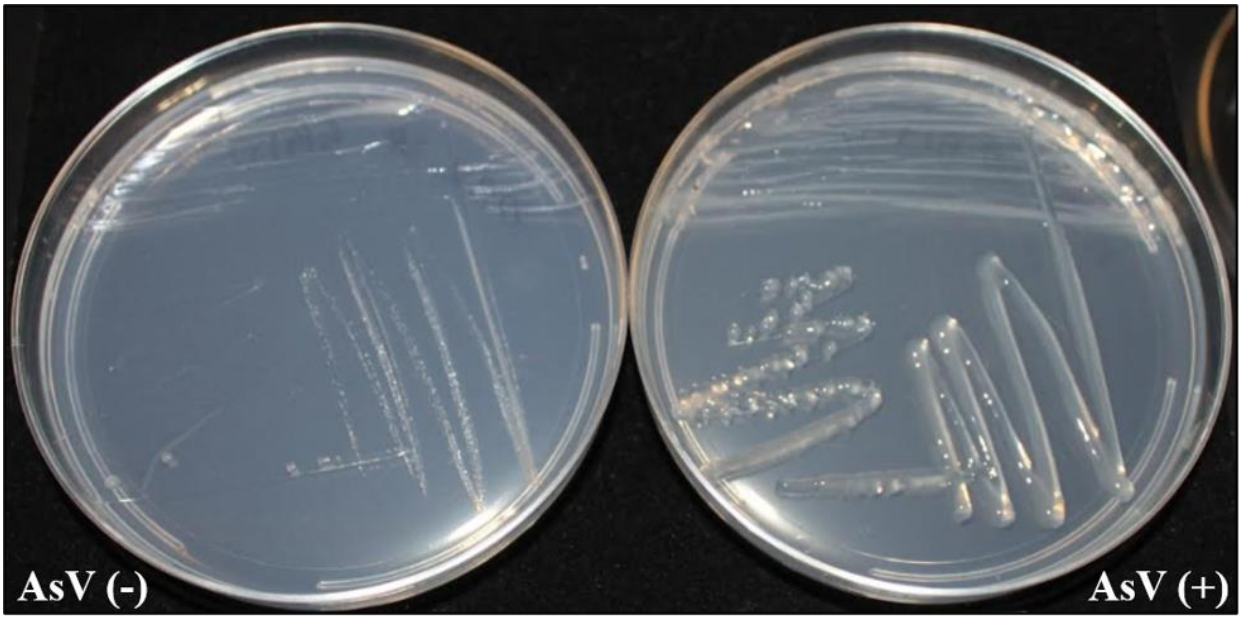

Photo 2. *Rahnella* PD12R on MS without AsV (left) and with 2 mM AsV (right) in the media. Shows biofilm formation under arsenic stress (48 hrs.)

Tables

Table 1. The mean root length (7 days) and mean biomass (14 days) of *A. thaliana* under arsenic stress. Surface-sterilized seeds inoculated with PD9R or PD12R and germinated on MS agar plates with 50 µM or 100 µM AsV. 10 seeds per plate, 4 plates per treatment (n = 4, ± SD).

PD9R			
[As]	Inoc.	Mean Root length (mm)	Mean Mass (dry, mg.)
Control	-E	16.93 ± 4.3	6.75 ± 4.0
Control	+E	19.62 ± 3.0	4.85 ± 1.1
50 µM	-E	12.21 ± 2.8	2.9 ± 1.8
50 µM	+E	13.40 ± 4.0	6.98 ± 1.6
100 µM	-E	2.58 ± 1.0	0.70 ± 0.2
100 µM	+E	9.98 ± 2.7	3.13 ± 0.7
PD12R			
[As]	Inoc.	Mean Root length (mm)	Mean Mass (dry, mg.)
Control	-E	9.40 ± 4.71	3.48 ± 2.2
Control	+E	10.78 ± 5.2	3.20 ± 1.0
50 µM	-E	7.95 ± 5.3	2.23 ± 1.0
50 µM	+E	8.50 ± 5.5	2.48 ± 0.9
100 µM	-E	5.4 ± 3.1	1.28 ± 0.4
100 µM	+E	9.33 ± 4.3	1.55 ± 0.2

Table 2. Identification of PD9R and PD12R via pairwise alignments with whole genome CDS for strains published in NCBI/RefSeq database (as of May 2020). The gANI score is derived from the average nucleotide identity of orthologous genes and the AF is a measure of the alignment fraction of those genes. The threshold scores for gANI and AF are 96.5% and 0.60, respectively. Shown are the top four matches for each strain.

Pseudomonas sp. PD9R		gANI		AF	
GenBank Accession	Strain	Fwd.	Rev.	Fwd.	Rev.
GCF_001952935.1	*Pseudomonas fredericksbergensis* sp. AS1	87.23	87.23	0.65	0.74
GCF_009866745.1	*Pseudomonas* sp. S34	86.99	86.99	0.70	0.78
GCF_000316175.1	*Pseudomonas* sp. UW4	86.91	86.91	0.69	0.77
GCF_0.003151075.1	*Pseudomonas* sp. 31-12	86.82	86.83	0.71	0.74
Rahnella sp. PD12R		gANI		AF	
GenBank Accession	Strain	Fwd.	Rev.	Fwd.	Rev.
GCF_002951615.1	*Rahnella* sp. ERMR1:05	93.21	93.21	0.81	0.82
GCF_003956145.1	*Rahnella aquatilis* KM05	85.99	86.01	0.73	0.81
GCF_003956105.1	*Rahnella aquatilis* KM12	85.98	85.99	0.72	0.82
GCF_003956125.1	*Rahnella aquatilis* KM25	85.98	85.99	0.72	0.82

Figures

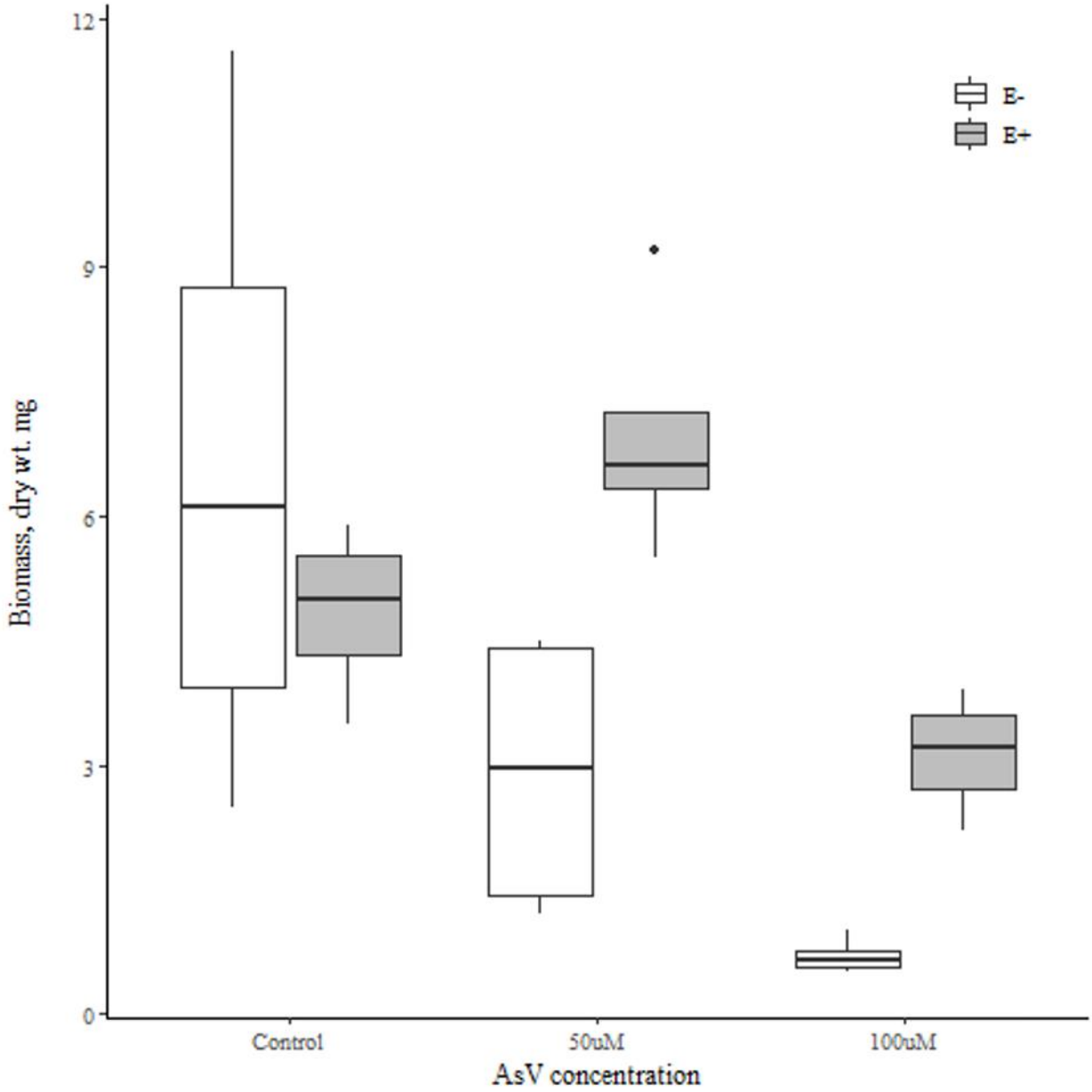

Figure 1. Mean biomass of *A. thaliana* inoculated with *Pseudomonas* sp. PD9R and grown on MS agar with AsV (50 µM or 100 µM). Inoculation with PD9R had a positive effect on biomass when AsV was in the media (Two-way ANOVA, $F_{2,18} = 4.58$, $p = 0.024$). The biomass of the PD9R inoculated plants was greater when under AsV stress as compared to the uninoculated controls (Tukey's HSD, $F_{1,6} = 11.68$, $p = 0.014$ and $F_{1,6} = 39.48$, $p < 0.001$, 50 µM and 100 µM, respectively). Inoculated plants (+E), mock-inoculated (- E), controls (0 AsV). 10 seeds per plate, 4 plates per treatment (n = 4).

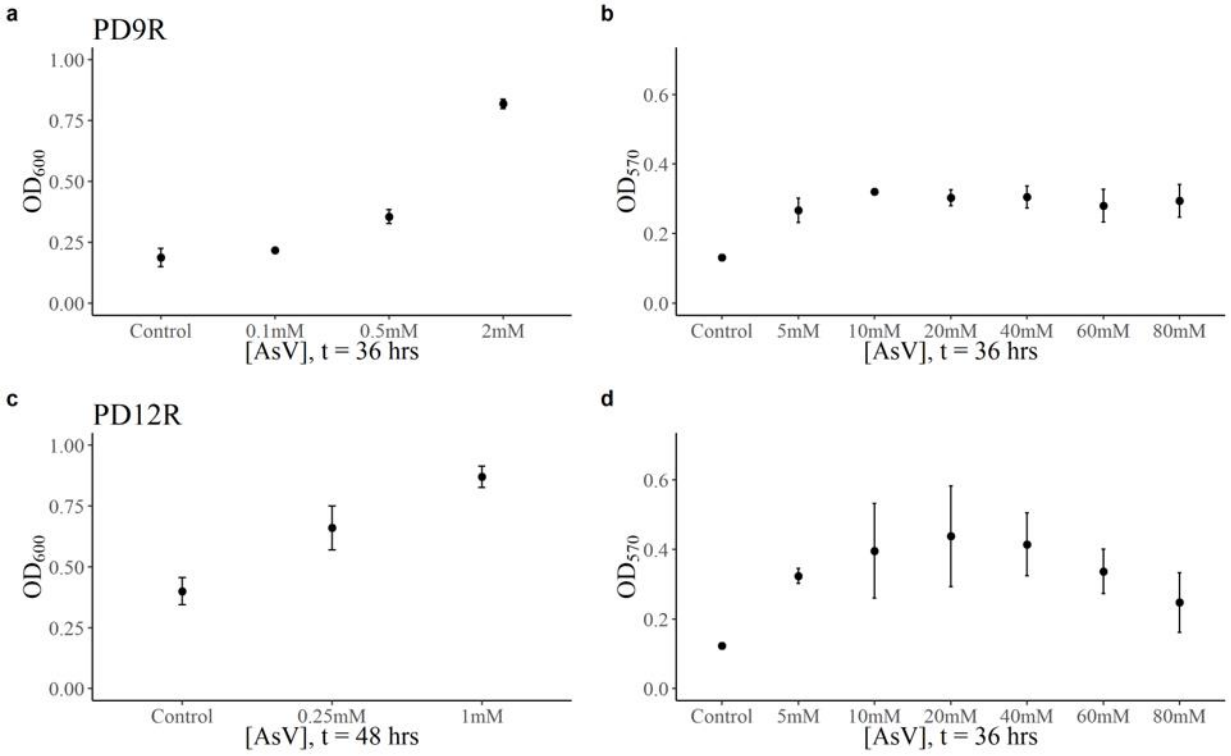

Figure 2. Mean OD for PD9R and PD12R experiments. Cells grown in MS media with AsV had higher OD readings than the controls (0 AsV). At low [AsV], OD increased as [AsV] increased in the media. (a) Pd9R: [AsV] (0 – 2 mM AsV) at 36 hrs. (ANOVA, $F_{2,6} = 1536.0$, $p < 0.001$), and (c) PD12R: [AsV] (0 – 1 mM AsV) at 48 hrs. ($F_{2,6} = 38.08$, $p < 0.001$). At higher AsV concentrations, the OD readings for the cells grown in AsV were different than the controls (0 AsV). (b) PD9R [AsV] (0 – 80 mM) at 36 hrs. (ANOVA $F_{6,14} = 11.98$, $p < 0.001$). Controls < AsV with no differences between treatments ($\alpha = 0.05$), and (d) PD12R: [AsV] (0 – 80 mM) at 36 hrs. ($F_{6,14} = 4.28$, $p = 0.012$). Controls ~ 5 mM ~ 80 mM and controls < 10 mM ~ 20 mM ~ 40 mM ~ 60 mM. ($\alpha = 0.05$). n = 3, ± SD for all experiments

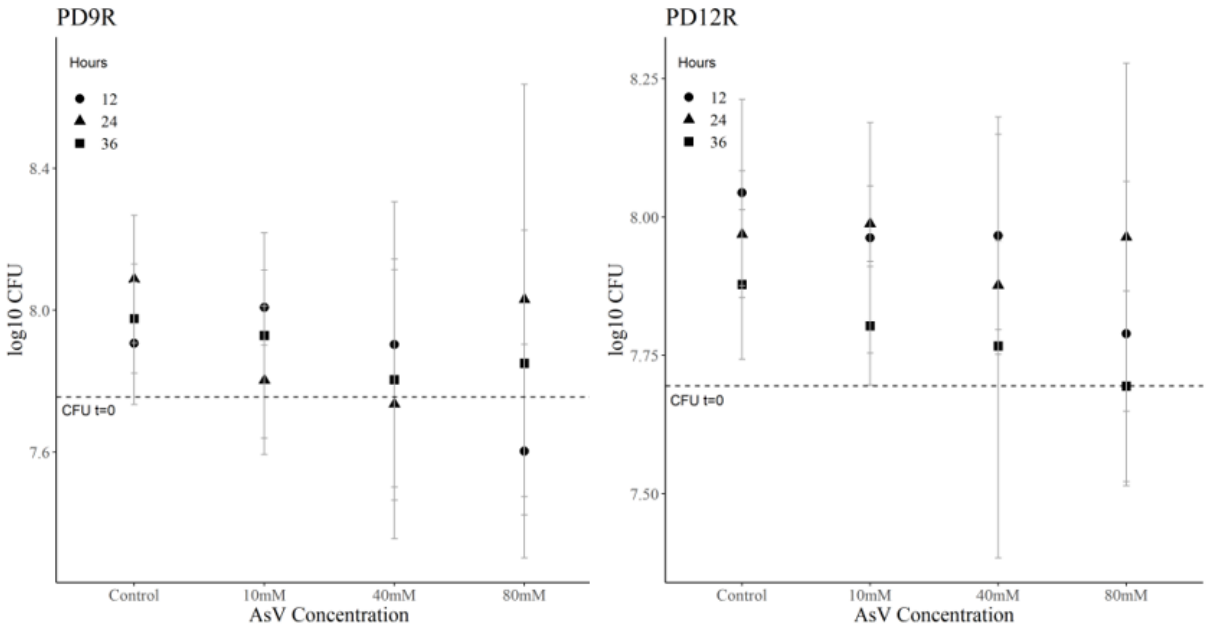

Figure 3. Mean CFU readings for PD9R (left) and PD12R (right) in MS with a range of AsV (0 mM – 80 mM). While the CFU counts trended down for both PD9R and PD12R, there was no effect of AsV concentration on CFU for either strain (repeated-measures ANOVA, $F_{3,32}$ = 1.405, p = 0.259 and $F_{3,32}$ = 1.755, p = 0.176 for PD9R and PD12R, respectively). n = 9, ±SD

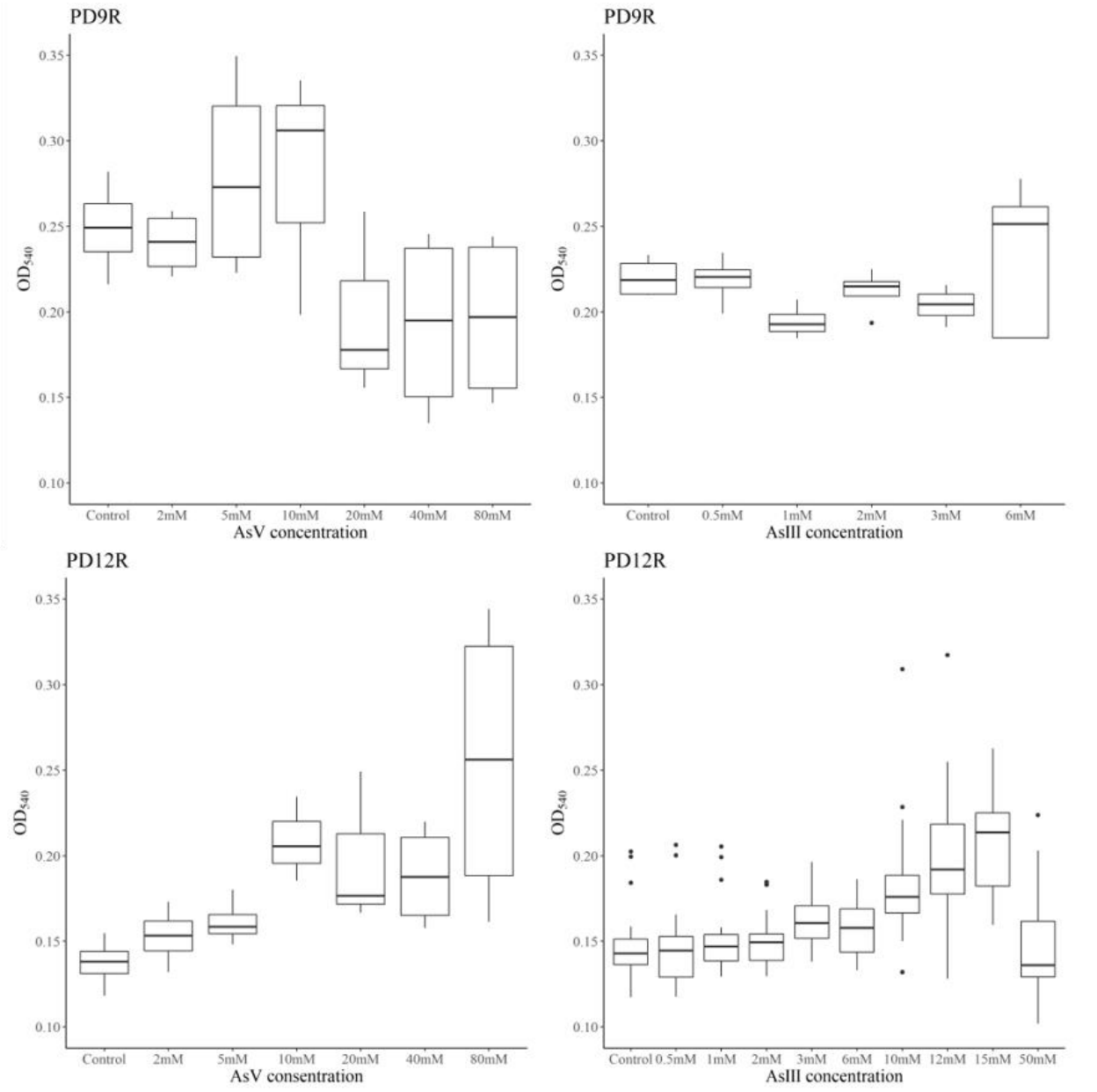

Figure 4. Biofilm formation by PD9R and PD12R in arsenic.

Top: PD9R biofilms were similar at lower AsV and AsIII concentrations, whether arsenic was in the media or not ($F_{9,230}$ = 9.19, $p < 0.0001$ and $F_{9,230}$ = 72.93, $p < 0.0001$ for AsV and AsIII, respectively). At higher concentrations PD9R's ability to form biofilms declined in both AsV (> 10 mM) and AsIII, with more than 50% failure at 10 mM and 12 mM and 100% failure at 15 mM and 50 mM (AsIII data not shown). n = 24

Bottom: PD12R biofilm formation increased with both AsV and AsIII, except for 50 mM AsIII ($F_{9,230}$ = 29.94, $p < 0.0001$ and $F_{9,230}$ = 20.13, $p < 0.0001$ for AsV and AsIII, respectively). n = 24

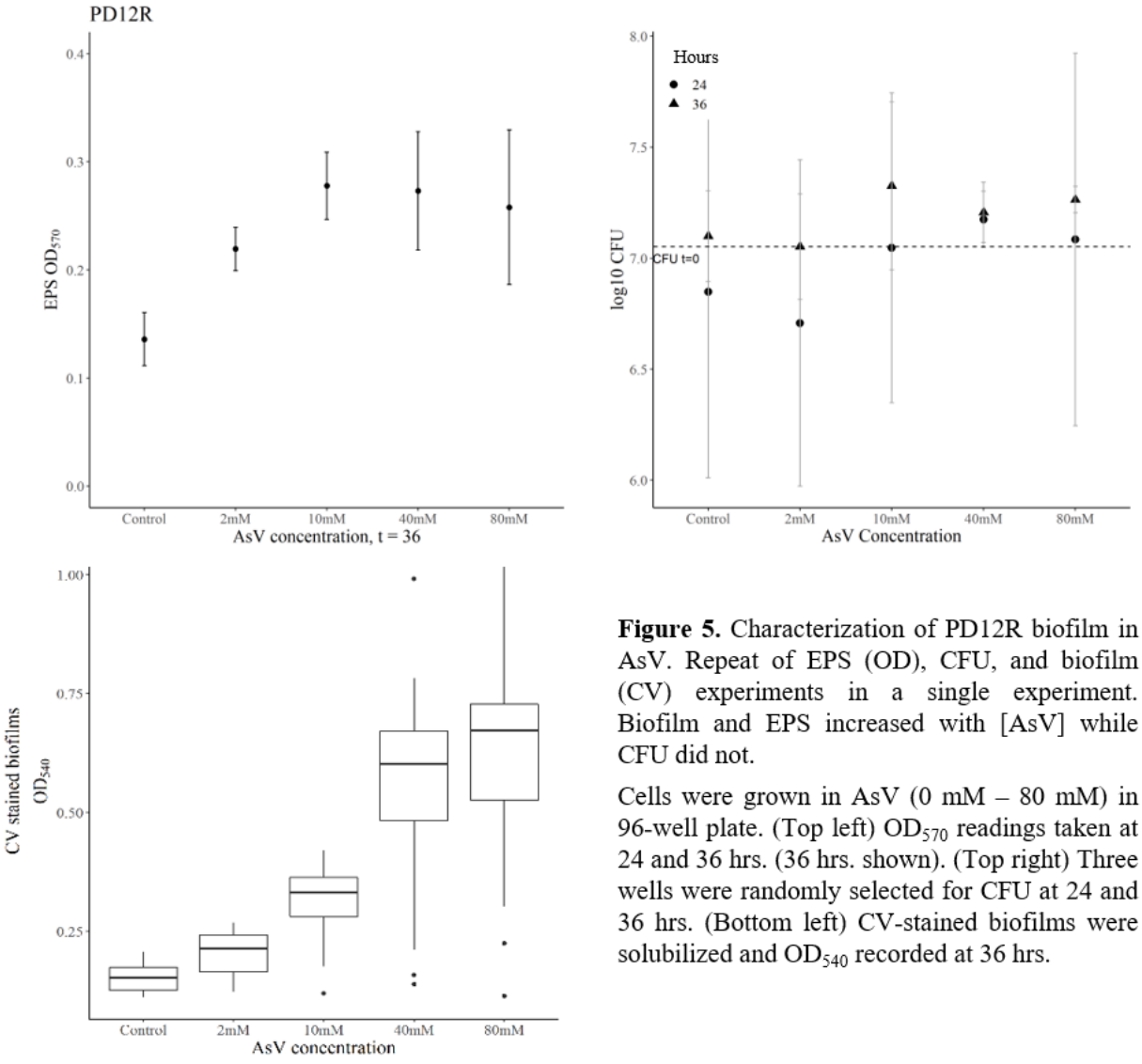

Figure 5. Characterization of PD12R biofilm in AsV. Repeat of EPS (OD), CFU, and biofilm (CV) experiments in a single experiment. Biofilm and EPS increased with [AsV] while CFU did not.

Cells were grown in AsV (0 mM – 80 mM) in 96-well plate. (Top left) OD_{570} readings taken at 24 and 36 hrs. (36 hrs. shown). (Top right) Three wells were randomly selected for CFU at 24 and 36 hrs. (Bottom left) CV-stained biofilms were solubilized and OD_{540} recorded at 36 hrs.

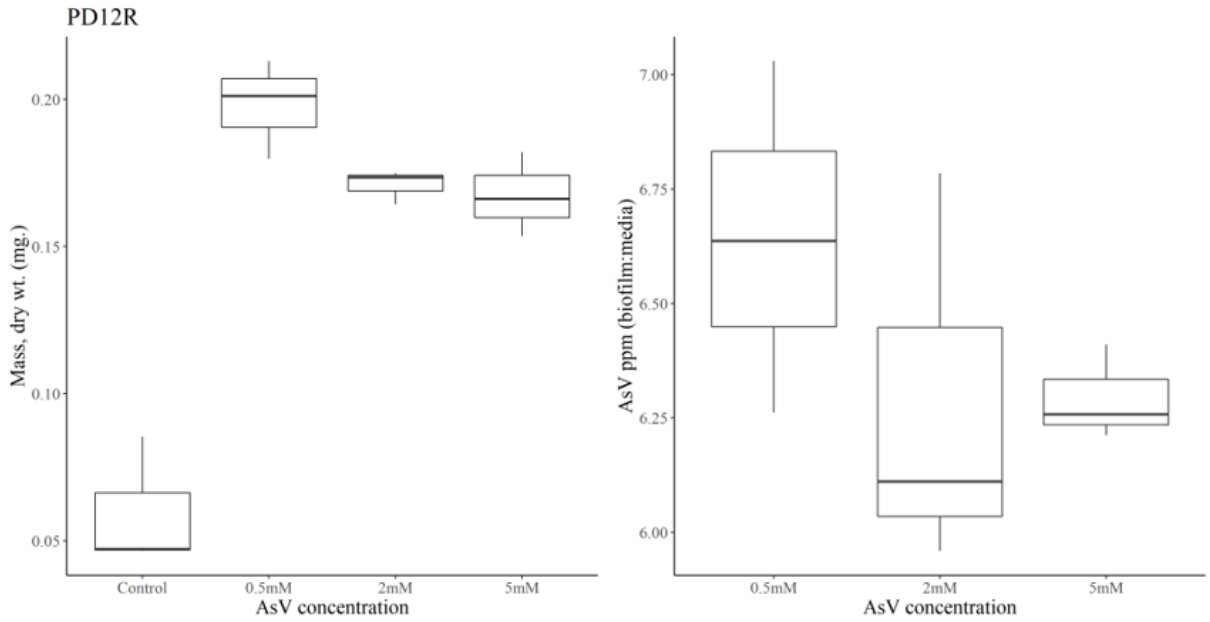

Figure 6. PD12R biofilms with AsV (0 mM – 5 mM). Biofilms were grown on 0.22 µM membranes placed on MS with AsV to capture both soluble and non-soluble components of biofilm.

(Left): The mean mass was greater when AsV was in the media, but not different between [AsV] ($F_{3,8} = 44.06$, $p < 0.0001$) (n=3).

(Right): AsV (ppm) in the biofilms was quantified via ICP-OES. When the biofilm [AsV] was normalized for the original [AsV] in the media, the AsV ppm in the biofilms was constant, ($F_{2,6} = 1.07$, $p = 0.40$, $n = 3$).

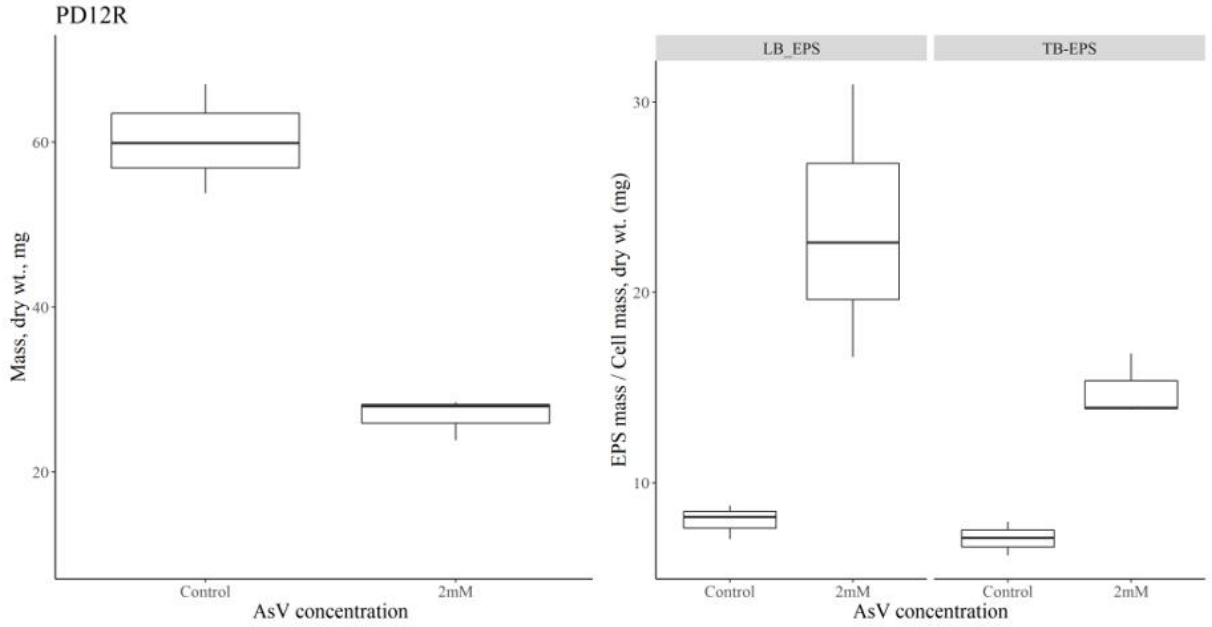

Figure 7. PD12R biofilm mass by soluble (LB-EPS), non-soluble (TB-EPS), and cell fraction.

(Left) Cells grown in 2 mM AsV had reduced mass (mg) as compared to controls ($t = 8.19$, $p = 0.007$). ($n = 3$) (Right) The AsV stressed cells produced more LP-EPS ($t = 3.67$, $p = 0.03$) and TB-EPS ($t = 7.12$, $p = 0.003$) than the control cells (w / w). $n = 3$

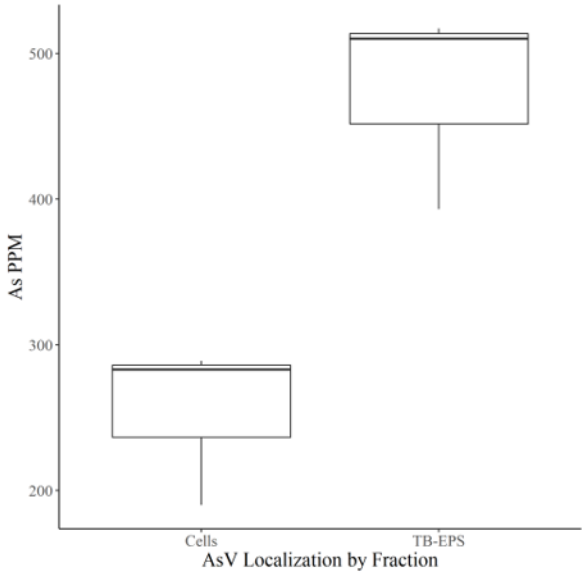

Figure 8. The mean AsV (ppm) associated with the cell and TB-EPS fractions of PD12R biofilms. The AsV accumulated in the TB-EPS by ~ 2:1 ratio ($t = 4.27$, $p = 0.007$) $n = 3$

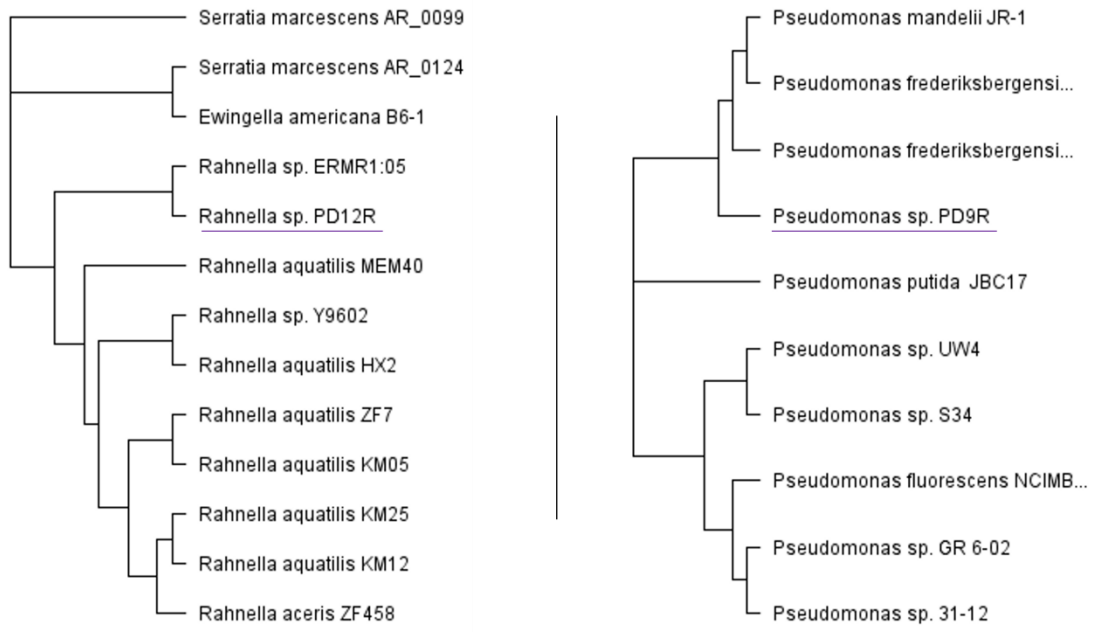

Figure 9. Phylogenetic tree of the 16s rRNA sequences *Rahnella* sp. PD12R and *Pseudomonas* sp. PD9R and reference genomes from NCBI/Ref.Seq. database (NTS). Constructed in Geneious Prime with the Neighbor-Joining algorithm using the Tamura-Nei genetic distance model.

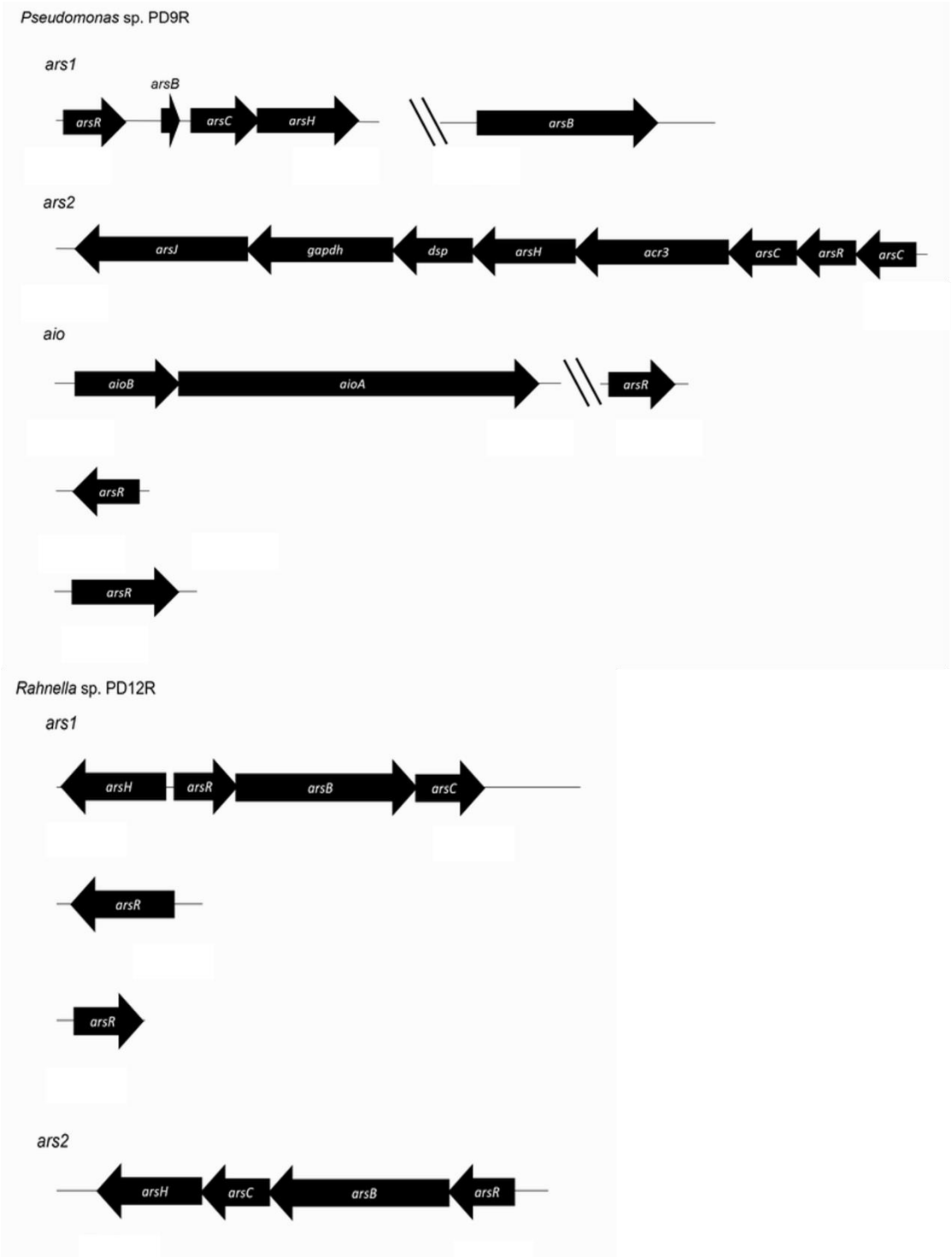

Figure 10. The arsenic detoxification genes from the genomes of *Pseudomonas* sp. PD9R and *Rahnella* sp. PD12R. (Top) PD9R contains two *ars* operons and a *aio* operon. The *ars2* operon contains the gene cluster *gapdh-arsJ* the only known arsenate efflux permease (Chen et at. 2016). All three identified *arsC* genes are from the thioredoxin family. (Bottom) PD12R contains two *ars* operons, both containing *arsC* from the glutaredoxin family.

Supplementary Tables

Table S1: Endophyte library, including host plant. Collected near Pt. Defiance Park, Tacoma, Pierce County, WA (47° 18' 57.3" N 122° 31' 41.0" W), July 2013.

	AsV concentration							
Strain	500uM	1 mM	2 mM	3 mM	4 mM	Family	Species	Common
PD 1 S	X					Ericaceae	*Gaultheria shallon*	Salal
PD 1 R	X					Ericaceae	*Gaultheria shallon*	Salal
PD 2 S	X					Berberidaceae	*Mahonia aquifolium*	Oregon Grape
PD 2 R	X					Berberidaceae	*Mahonia aquifolium*	Oregon Grape
PD 3 S	X					Dryopteridaceae	*Polystichum munitum*	Swordfern
PD 3 R	X					Dryopteridaceae	*Polystichum munitum*	Swordfern
PD 4 S	X					Ericaceae	*Vaccinium ovatum*	Evergreen Huckleberry
PD 4 R	X					Ericaceae	*Vaccinium ovatum*	Evergreen Huckleberry
PD 5 S	X	X	X	X	X	Equisetaceae	*Equisetum arvense*	Common Horsetail
PD 5 R	X	X	X	X	X	Equisetaceae	*Equisetum arvense*	Common Horsetail
PD 6 S	X					Dryopteridaceae	*Polystichum munitum*	Swordfern
PD 6 R	X					Dryopteridaceae	*Polystichum munitum*	Swordfern
PD 7 S	X	X	X	X	C	Rosaceae	*Rubus parviflorus*	Thimbleberry
PD 7 R	X	X	X	X	X	Rosaceae	*Rubus parviflorus*	Thimbleberry
PD 8 S	X					Dennstaedtiaceae	*Pteridium aquilinium*	Bracken
PD 8 R	X					Dennstaedtiaceae	*Pteridium aquilinium*	Bracken
PD 9 S	X	X	X	X	X	Rosaceae	*Rubus spectabilis*	Salmonberry
PD 9 R	X	X	X	X	X	Rosaceae	*Rubus spectabilis*	Salmonberry
PD 10 S	X	X	X			Dennstaedtiaceae	*Pteridium aquilinium*	Bracken
PD 10 R	X	C	X	X	X	Dennstaedtiaceae	*Pteridium aquilinium*	Bracken
PD 11 S	X					Pinaceae	*Tsuga heterophylla*	Western Hemlock
PD 11 R	X	X	X			Pinaceae	*Tsuga heterophylla*	Western Hemlock
PD 12 S	X	X	X	X	X	Ericaceae	*Vaccinium parvifolium*	Red Huckleberry
PD 12 R	X	X	X	X	X	Ericaceae	*Vaccinium parvifolium*	Red Huckleberry

S = shoot, R = root, and C = contaminated samples.

Table S1 (cont.): Endophyte library, including host plant. Collected near Maury Island Marine Park, Vashon, King County, WA (47° 22' 44.8" N 122° 24' 19.8" W), July 2013.

	AsV concentration							
Strain	500uM	1 mM	2 mM	3 mM	4 mM	Family	Species	Common
VM 1 S	X					Dryopteridaceae	*Polystichum munitum*	Swordfern
VM 1 R	X	X	X	X		Dryopteridaceae	*Polystichum munitum*	Swordfern
VM 2 S	X	X	X	C		Rosaceae	*Physiocarpus capitatus*	Pacific ninebark
VM 2 R	X					Rosaceae	*Physiocarpus capitatus*	Pacific ninebark
VM 3 S	X	X	X	X	X		*unidentified*	Unknown grass
VM 3 R	X	X	X	X	X		*unidentified*	Unknown grass
VM 4 S	X					Rosaceae	*Physiocarpus capitatus*	Pacific ninebark
VM 4 R	X					Rosaceae	*Physiocarpus capitatus*	Pacific ninebark
VM 5 S	X					Rosaceae	*Sorbus scopulina*	Western mountain ash
VM 5 R	X	X	X			Rosaceae	*Sorbus scopulina*	Western mountain ash
VM 6 S	X	X	X			Dryopteridaceae	*Polystichum munitum*	Swordfern
VM 6 R	X					Dryopteridaceae	*Polystichum munitum*	Swordfern
VM 7 S	X					Pinaceae	*Picea spp.*	non-native spruce
VM 7 R	X					Pinaceae	*Picea spp.*	non-native spruce
VM 8 S	X	X	X			Ericaceae	*Arctostaphylos uva-ursi*	Kinnikinnick
VM 8 R	X					Ericaceae	*Arctostaphylos uva-ursi*	Kinnikinnick
VM 9 S	X					Caprifoliaceae	*Symphoricarpos albus*	Snowberry
VM 9 R	X	C	X	X	X	Caprifoliaceae	*Symphoricarpos albus*	Snowberry
VM 10 S	X						*unidentified*	unknown shrub
VM 10 R	X						*unidentified*	unknown shrub
VM 11 S	X					Ericaceae	*Gaultheria shallon*	Salal
VM 11 R	X	X	X			Ericaceae	*Gaultheria shallon*	Salal
VM 12 S	X					Sapindaceae	*Acer circinatum*	Vine maple
VM 12 R	X	C	X	X		Sapindaceae	*Acer circinatum*	Vine maple
VM 13 S	X					Dennstaedtiaceae	*Pteridium aquilinium*	Bracken
VM 13 R	X	X	X	X	X	Dennstaedtiaceae	*Pteridium aquilinium*	Bracken
VM 14 S	X					Urticaceae	*Urtica dioica*	Stinging Nettle
VM 14 R	X					Urticaceae	*Urtica dioica*	Stinging Nettle
VM 15 S	X	X	X	X	X	Ericaceae	*Gaultheria shallon*	Salal
VM 15 R	X					Ericaceae	*Gaultheria shallon*	Salal
VM 16 S	X					Ericaceae	*Arbutus menziesii*	Pacific madrona
VM 16 R	X					Ericaceae	*Arbutus menziesii*	Pacific madrona

S = shoot, R = root, and C = contaminated samples.