

Effect of Arsenic on EPS Synthesis, Biofilm Formation, and Plant Growth-Promoting Abilities of the Endophytes *Pseudomonas* PD9R and *Rahnella laticis* PD12R

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ABSTRACT: Phytoremediation, a cost-effective, eco-friendly alternative to conventional remediation, could expand efforts to remediate arsenic-contaminated soils. As with other pollutants, the plant microbiome may improve phytoremediation outcomes for arsenic-contaminated sites. We used *in vitro* and *in silico* methods to compare the arsenic resistance mechanisms, synthesis of extracellular polymeric substances (EPS), biofilm formation, and plant growth-promoting abilities of the endophytes *Pseudomonas* sp. PD9R and *Rahnella laticis* PD12R. PD12R, which tolerates arsenate (As(V)) and arsenite (As(III)) to concentrations fivefold greater than PD9R, synthesizes high volumes of EPS in response to arsenic, and sequesters arsenic in the capsular EPS and cells. While arsenic exposure induced EPS synthesis in both strains, only PD12R continued to form biofilms at high As(III) and As(V) concentrations. The effects of endophyte inoculation on *Arabidopsis* growth varied by strain and As(V) concentration, and PD9R had positive effect on plants exposed to low levels of arsenic. Comparative genomic analyses exploring the EPS synthesis and arsenic resistance mechanisms against other *Pseudomonas* and *Rahnella* strains suggest that both strains possess atypical arsenic resistance mechanisms from other plant-associated strains, while the configuration of the EPS synthesis systems appeared to be more broadly distributed among plant- and non-plant-associated strains.

KEYWORDS: phytoremediation, bioremediation, *Rahnella*, *Pseudomonas*, environmental restoration, arsenic resistance, biofilms, extracellular polymeric substances



INTRODUCTION

Arsenic is a pollutant of global concern due to its toxicity at low concentrations and persistence in the environment. Historical sources of anthropogenic arsenic pollution, such as mining, industry, and agriculture, have increased soil arsenic concentrations in many areas by orders of magnitude over background levels, and while arsenic production declined in the latter half of the 20th century, the reductions were largely replaced by arsenic emissions from increased fossil fuel consumption,¹ the mining and smelting of other metals,^{2,3} and its use in modern electronics.⁴ While effective, conventional remediation strategies for arsenic-contaminated soils, such as excavation or capping, are energy-intensive, produce secondary pollution, and expensive, and have limited clean-up efforts to only the most severely contaminated sites.^{5,6} Consequently, moderately contaminated sites remain unremediated, increasing risk of public exposure and reducing economic productivity of the affected lands.

Phytoremediation is a low-cost, eco-friendly technology that could extend the reach of conventional remediation efforts for arsenic-contaminated soils. For example, phytostabilization, the containment of arsenic in the plant roots and rhizosphere, and

phytoextraction, the accumulation of arsenic in the harvestable tissues, are less expensive to implement, require fewer energy and chemical inputs, and provide important ecosystem services.^{7–9} However, phytoremediation is slower and less predictable than conventional remediation technologies, and its effectiveness can be reduced by phytotoxicity.^{7,10} Initial approaches to address these challenges were plant-focused, i.e., plant selection or genetic transformation. More recently, the influence of the plant microbiome on host fitness and adaptation has received greater recognition, and motivated research into the potential of plant-associated microorganisms to improve the phytoremediation of many pollutants, including arsenic.

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Plant-associated microorganisms promote host tolerance to a range of environmental stressors, including low-nutrient soils, drought, pathogens, and toxins.^{11,12} Endophytes, microorganisms colonizing the internal tissues of plants, may assist phytoremediation indirectly through various plant growth-promoting (PGP) traits, e.g., phytohormone synthesis, improved nutrient acquisition, modulation of the host stress response, and pathogen suppression, or they may assist directly by reducing the phytotoxicity of the pollutant. With inorganic pollutants, this includes degrading the pollutant in planta, as has been demonstrated with toluene,¹³ 2,4-D,¹⁴ PAHs,¹⁵ and TCE.¹⁶ However, inorganic pollutants, such as arsenic, cannot be degraded and potential direct mechanisms should promote the accumulation of arsenic in the harvestable tissues.

The most common forms of inorganic arsenic available for uptake by plants and microorganisms are arsenate As(V) and arsenite As(III), the dominant species in aerobic and anaerobic conditions, respectively.^{17–20} As(V), a structural and chemical analogue of phosphate, is acquired via the phosphate transport system, while As(III), the more toxic of the two species, enters cells through aquaglyceroporins.^{20–22} The most widely distributed arsenic resistance pathway, identified in bacteria,²³ yeast,²⁴ and plants,^{25,26} involves the cytoplasmic reduction of As(V) to As(III) via arsenate reductase enzymes, followed by efflux from the cell or chelation with metal-binding ligands, such as glutathione or phytochelatin.^{17,27} Interestingly, the high affinity of As(III) for sulfhydryl (–SH) groups in catalytic enzymes explains both its toxicity and detoxification. Despite As(III)'s increased toxicity, this pathway is considered a detoxification mechanism that confers resistance to both arsenic species by protecting phosphate metabolism from As(V) attacks and maintaining cellular phosphate levels, while decreasing arsenic levels through the efflux of As(III) instead of As(V).²⁸

Bacterial endophytes have various mechanisms that act directly on arsenic to decrease toxicity or reduce bioavailability, e.g., transformation, precipitation, adsorption, sequestration, or biomethylation.¹⁸ In *Solanum nigrum*, an endophyte consortium expanded glutathione pools and increased arsenate reductase activities, improving arsenic transformation levels by 33% in the inoculated plants.²⁹ Another potential direct mechanism is sequestration in biofilms, aggregates of bacterial cells embedded in a matrix of extracellular polymeric substances (EPS), protecting the cells from biotic and abiotic stresses.³⁰ The EPS matrix is composed of various polysaccharides, proteins, nucleic acids, lipids, eDNA, and other components rich in charged functional groups with potential binding sites for heavy metal(loid) ions,^{31,32} creating distinct physiochemical conditions that induce metabolic activities not found in free-living planktonic cells.³⁰ Bacterial biofilms are effective in the adsorption of V, Ni, and Cu from fly ash,³³ Cd from aqueous solutions,³⁴ and oxidizing arsenite (As(III)) to (As(V)) in acid mine drainage.³⁵ Although bacterial cells are more likely to be associated with biofilms than as free-living planktonic cells, few studies have considered the potential of endophytic biofilms to contribute to arsenic detoxification.

In this study, we isolated the endophytes, *Pseudomonas* sp. PD9R and *Rahnella laticis* PD12R, from native plants growing in arsenic-contaminated soils and screened them for arsenic resistance and the ability to promote arsenic tolerance in *Arabidopsis thaliana*. We also investigated the induction of EPS synthesis and biofilm formation by As(V) and arsenite (As(III)). The sequenced genomes were mined for genes

associated with arsenic detoxification and EPS synthesis and subjected to comparative analyses with other plant-associated *Rahnella* and *Pseudomonas* strains.

METHODS AND MATERIALS

Detailed Methods. Detailed methods are provided in the Supporting Information (File S1).

Isolation of Endophytes. The endophytes were isolated from native plants growing on undisturbed forest soils located within an area of known arsenic contamination (Table S1). Root and shoot tissues from 38 plants were surface-sterilized per Doty and colleagues (2009).³⁶ The sterilized tissues were macerated in 10 mL of Murashige & Skoog Basal Medium (MS), and 100 μ L of the plant extracts was plated on MS agar with 500 μ M As(V). Following incubation, 30 morphologically distinct colonies were streak-purified three times, and the isolates were further screened on MS agar with As(V) (1, 2, and 4 mM). To estimate the minimum bactericidal concentration (MBC) of arsenic, 100 μ L aliquots of inoculum for each strain were incubated, in triplicate, in 96-well plates in As(V) (25–500 mM) or As(III) (1–20 mM), and the presence or absence of colonies on Mannitol Glutamate/Luria-Bertani (MG/L) recorded after 24 h incubation.

Inoculation of a Novel Host, *A. thaliana*. Surface-sterilized *A. thaliana* seeds (ecotype Col-0) were inoculated with 1 mL of inoculum from PD9R, PD12R, or mock-inoculated in sterile MS media. After 24 h, 10 seeds were transferred onto MS agar with As(V) (0 mM, 50 μ M, 100 μ M), and the Parafilm sealed plates placed on edge in a growth chamber (16 h photoperiod, 150 μ mol m^{–2}, 23 °C). At 7 days, the plates were photographed, and the root lengths (mm) measured using the *Line Selection Tool* option in ImageJ,³⁷ and at 14 days, the plants were destructively harvested, dried for 24 h at 80 °C, and biomass (mg, dry wt) was recorded. Each group consisted of four plates with 10 seeds per plate, and the root length and biomass were estimated using the cumulative sums per plate, divided by the number of plates in the group ($n = 4$).

Optical Density Measurements during Growth in Arsenic. The strains were cultured in 10 mL of MS with 100 μ M, 500 μ M, and 2 mM As(V) for PD9R, and 250 μ M and 1 mM As(V) for PD12R, and the OD₆₀₀ recorded at 48 h. A follow-up experiment was conducted using 100 μ L of MS with As(V) (5–80 mM) in 96-well plates, and the OD₆₂₀ was recorded after 24 and 36 h. These experiments were performed in triplicate and included 0 As(V) controls.

Colony-Forming Units (CFU). To estimate viable cells for PD9R and PD12R cultured in As(V) (0 to 80 mM), 2 mL of inoculum was cultured in 12-well plates, with nine replicates per concentration ($n = 9$). At 36 h, 100 μ L aliquots of serial dilutions (10^{–4}–10^{–6}) were plated on MG/L agar and the CFUs counted after 24 h.

Biofilm Formation under Arsenic Stress. To estimate PD9R and PD12R biofilm formation in the presence of arsenic, 0.1% crystal violet (CV) solution was used to stain the biofilms as described by Merritt and colleagues.³⁸ The biofilms were grown for 48 h under static conditions with As(V) (0 to 80 mM) or As(III) (0 to 50 mM) and stained with 0.1% CV, after which the CV-stained biofilms were solubilized in 30% acetic acid and the OD₅₄₀ was recorded. The experiments were repeated three times with eight replicates per concentration ($n = 24$).

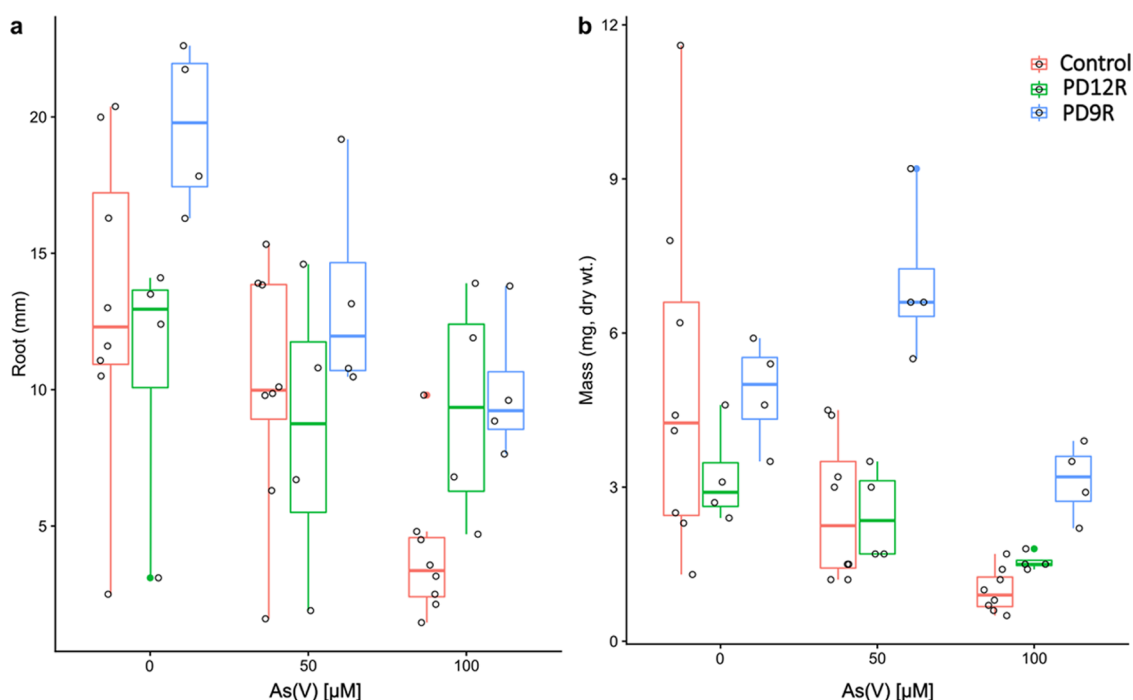


Figure 1. Effect of endophyte inoculation on (a) root length at 7 days and (b) total biomass at 14 days for *A. thaliana* exposed to As(V) (0, 50, and 100 μM). Compared to the uninoculated plants, PD9R increased root length and biomass ($P = 0.001$ and $P = 0.004$), while PD12R had no effect ($P = 0.786$ and $P = 0.495$). Two-factor ANOVA with interaction between endophyte and As(V), ten seeds per plate, four plates per treatment ($n = 4$). Open dots (○) represent actual data points used in the calculation of box plots (offset for visibility), and the closed dots (●) represent data points greater than $1.5 \times \text{IQR}$.

Arsenic Adsorption and Localization in PD12R Biofilms: Arsenic Adsorption and Localization in PD12R Biofilms. PD12R high quantities of viscous EPS on the surface of MS agar plates were supplemented with As(V). We compared the total EPS synthesized and arsenic adsorbed by PD12R biofilms grown on sterile polycarbonate membranes (Whatman, 0.22 μM, 19 mm) set onto MS agar supplemented with 0–5 mM As(V) as described by Merritt et al.³⁸ Six membranes per plate were inoculated with 15 μL aliquots of PD12R cell suspensions ($\text{OD}_{600} = 0.1$), with three plates per As(V) concentration ($n = 3$) (Figure S2). After 72 h incubation, the six membranes and biofilms were collectively weighed (mg, wet wt), subjected to heat/acid digest per EPA method 3050B (File S1), and the arsenic concentration (mg kg^{-1} , as parts per million, ppm) was estimated via inductively coupled plasma–optical emission spectrometry (ICP-OES, Thermo Fisher Scientific, Model 6300, SEFS Soil Analytics Lab, University of Washington). To account for the mass and arsenic concentration of the membranes, three plates with uninoculated membranes were treated identically to the inoculated plates and the mean mass and arsenic ppm of the inoculated plates were adjusted by means of the uninoculated plates.

A second experiment was conducted to determine where arsenic localizes in PD12R biofilms. PD12R was grown in 100 mL of MS supplemented with 2 mM As(V) for 48 h, and the resulting cell suspensions were separated into the soluble (S-EPS), bound (B-EPS), and cell fractions using physiochemical methods described by Comte and colleagues.³⁵ Briefly, the S-EPS fraction was extracted as the supernatant after centrifugation for 20 min at 10 000 RPM. The pellets, composed of the B-EPS and cell fractions, were resuspended by vortexing in sterile PBS and incubated with 2% EDTA (pH

8.0) for 3 h at 4 °C. Next, the B-EPS fraction was extracted as the supernatant after centrifugation for 20 min at 10 000 RPM. The remaining pellets were considered the cell fraction. The three fractions were lyophilized at -50 °C for 48 h, masses were recorded, and heat/acid-digested for ICP-OES analysis (EPA 3050B). For the S-EPS and B-EPS fractions, 250 mg of samples was digested, and the entire mass of the cell fraction was digested.

WGS Sequencing, Assembly, Annotation, and Taxonomic Classification. A shotgun library was constructed using Nextera DNA Flex (Illumina, Cat. No. 20018705) and sequenced at the University of Washington Northwest Genomics Center (NWGC) on an Illumina MiSeq platform (300-Cycle V2 Reagent Kit). The paired-end reads were assembled using the Geneious Prime assembler (v2020.0.3), and the draft assemblies were annotated via Rapid Annotation Subsystems Technology (RAST).³⁹ Functional annotation and KEGG Ortholog (KO) assignment were completed using KEGG KofamKOALA,⁴⁰ and whole-genome-based taxonomic classification and phylogenetic analysis via the Type (Strain) Genome Server (TYGS).⁴¹

Comparative Genomic Analysis of the Arsenic Resistance and EPS Genes. A dataset of plant-associated *Pseudomonas* and *Rahnella* strains was constructed with genomic assemblies obtained from the NCBI/RefSeq Assemblies database using the Advanced Search Builder tool.⁴² The searches yielded two datasets containing 27 *Pseudomonas* and 15 *Rahnella* strains, which were subjected to comparative analyses of the arsenic resistance mechanisms and EPS biosynthesis genes against PD9R and PD12R, respectively.

Comparative Genome Analysis of EPS Biosynthetic Genes in Plant-Associated *Rahnella* Strains. The

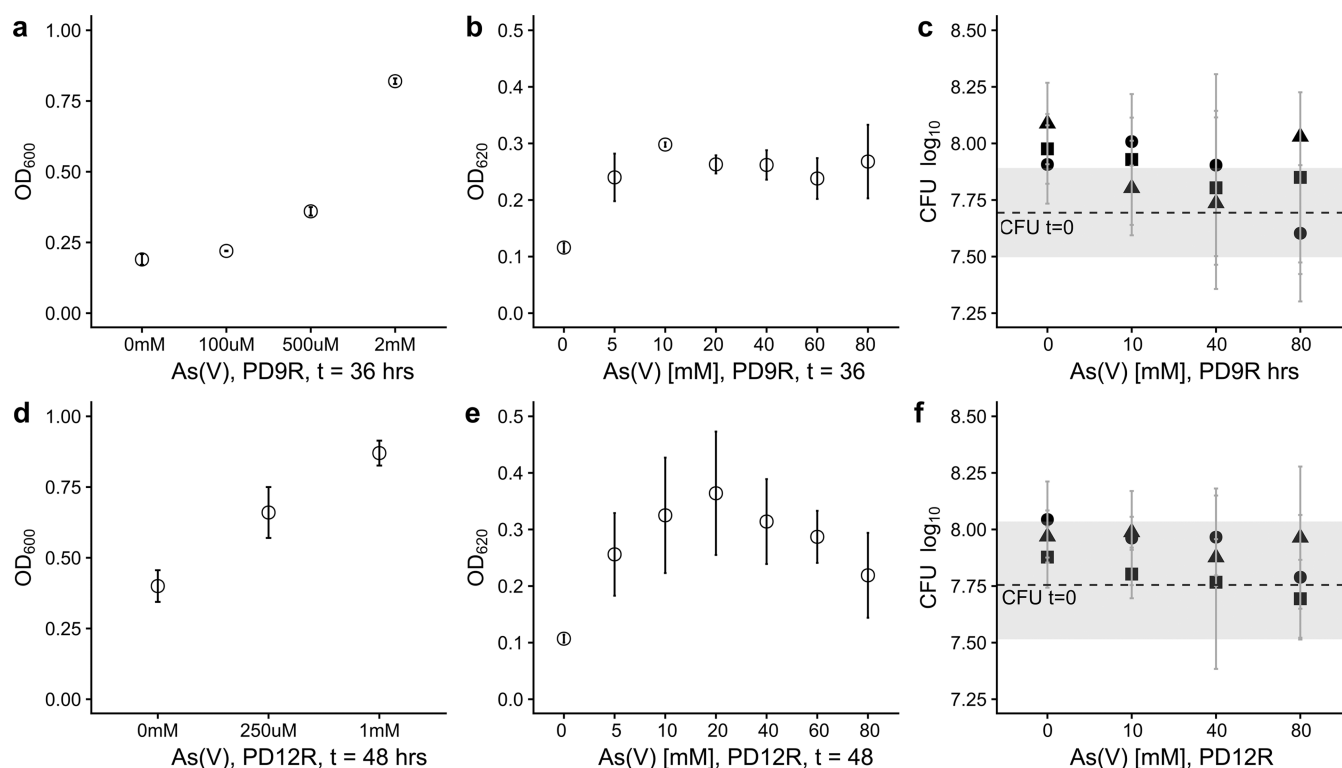


Figure 2. Exposure to As(V) increased EPS synthesis in PD9R and PD12R ($P < 0.05$) but had no effect on CFU count ($P > 0.05$). PD9R and PD12R OD readings at (a, d) 0 to 2 mM As(V) and (b, e) 0 to 80 mM As(V). One-way ANOVA ($n = 3$). (c, f) PD9R and PD12R CFU counts in 0 to 80 mM As(V). Log₁₀ CFU counts of aliquots collected at 12 h (●), 24 h (▲), and 36 h (■). The dashed line is log₁₀ CFU count at $t = 0 \pm 95\%$ CI. Repeated-measures ANOVA ($n = 9$). Bars represent standard deviation.

250 significant association of EPS biosynthetic genes in plant-
251 associated *Rahnella* strains was calculated using the Anvi'o 7.1
252 pan-genome pipeline.⁴³ The "anvi-compute-functional-enrich-
253 ment" program was applied to the *Rahnella* pan-genomes
254 database to identify functional orthologs (KEGG orthologs)
255 significantly associated with the plant-associated *Rahnella*
256 strains (Table S2).

257 **Statistics and Data Availability.** Statistical tests were
258 performed in R version 4.0.2 (R Core Team 2013), using one-
259 way or two-way analysis of variance (ANOVA), and Tukey's
260 HSD post hoc, as appropriate. The Newick files generated by
261 the TYGS pipeline were imported into iTOL ([https://itol.](https://itol.embl.de/)
262 [embl.de/](https://itol.embl.de/)) to construct the phylogenetic trees.⁴⁴ The PD9R
263 (GCA_019200975.1) and PD12R (GCA_019200965.1) se-
264 quence data were submitted to NCBI under BioProject
265 PRJNA742009.

266 ■ RESULTS AND DISCUSSION

267 **Isolation of Arsenic-Tolerant Endophytes.** We isolated
268 30 endophytes from plants growing on arsenic-contaminated
269 soils on MS agar supplemented with 500 μ M As(V), 14 of
270 which were tolerant to at least 4 mM As(V) (Table S1). The
271 root endophyte PD12R showed robust growth on the plates
272 and was observed to produce high volumes of EPS when
273 exposed to As(V) concentrations above 1 mM As(V). PD12R
274 was selected for further study, along with the strain PD9R,
275 which also exhibited robust growth on low levels of As(V) but
276 did not produce similar quantities of EPS. The strains were
277 identified via 16S rRNA sequence analysis as *Rahnella* sp.
278 PD12R and *Pseudomonas* sp. PD9R. Estimates of the minimum
279 bactericidal concentration (MBC) showed that PD12R was

resistant to arsenic concentrations fivefold greater than PD9R, 280
where PD9R failed to grow above 100 mM As(V) and 10 mM 281
As(III), PD12R grew at 500 mM As(V) and 50 mM As(III), 282
the highest concentrations evaluated. By comparison, Tashan 283
and colleagues⁴⁵ report the root endophyte *Pseudomonas* sp. 284
QNC1 tolerated 350 mM As(V), while the strains 285
Acinetobacter sp. QNA1 and QNA2, and *Rahnella* sp. QNC2 286
tolerated 300 mM As(V), while endophytes isolated from the 287
arsenic-hyperaccumulator *Pteris vittata*, which accumulates > 288
22 000 mg kg⁻¹ arsenic in its fronds,⁴⁶ tolerated at least 15 mM 289
As(III), while several strains grew up to 48 mM As(III).⁴⁷ 290

Inoculation of *A. thaliana* with PD9R and PD12R. In 291
the *A. thaliana* experiment, inoculation with PD9R improved 292
both root length ($P = 0.001$) and biomass ($P = 0.004$) in *A.* 293
thaliana, while PD12R had no effect on either root length or 294
biomass at the As(V) at the concentrations tested ($P > 0.05$) 295
(Figure 1a,b). As expected, As(V) had a negative effect on *A.* 296
thaliana in terms of both root length ($P = 0.001$) and biomass 297
($P = 0.001$). However, compared with the mean root length 298
and biomass results of the uninoculated plants, the effect of 299
inoculation with the arsenic-tolerant endophytes appeared to 300
increase with As(V) concentration for both strains (Table S3). 301
PD9R increased the mean root lengths by 49, 33, and 150% at 302
the 0, 50, and 100 μ M As(V) concentrations, respectively, and 303
while the PD12R inoculated plants had shorter mean root 304
lengths by 18 and 16% at 0 and 50 μ M As(V), respectively, the 305
mean root length of the PD12R inoculated plants exceed those 306
of the uninoculated plants by 137% at 100 μ M As(V). Biomass 307
followed a similar pattern, except in the case of the 0 μ M 308
As(V) group, where the mean biomass of the PD9R inoculated 309
plants was slightly less than the uninoculated plants (4.85 vs 310
5.03 mg). When the plants were exposed to As(V), the mean 311

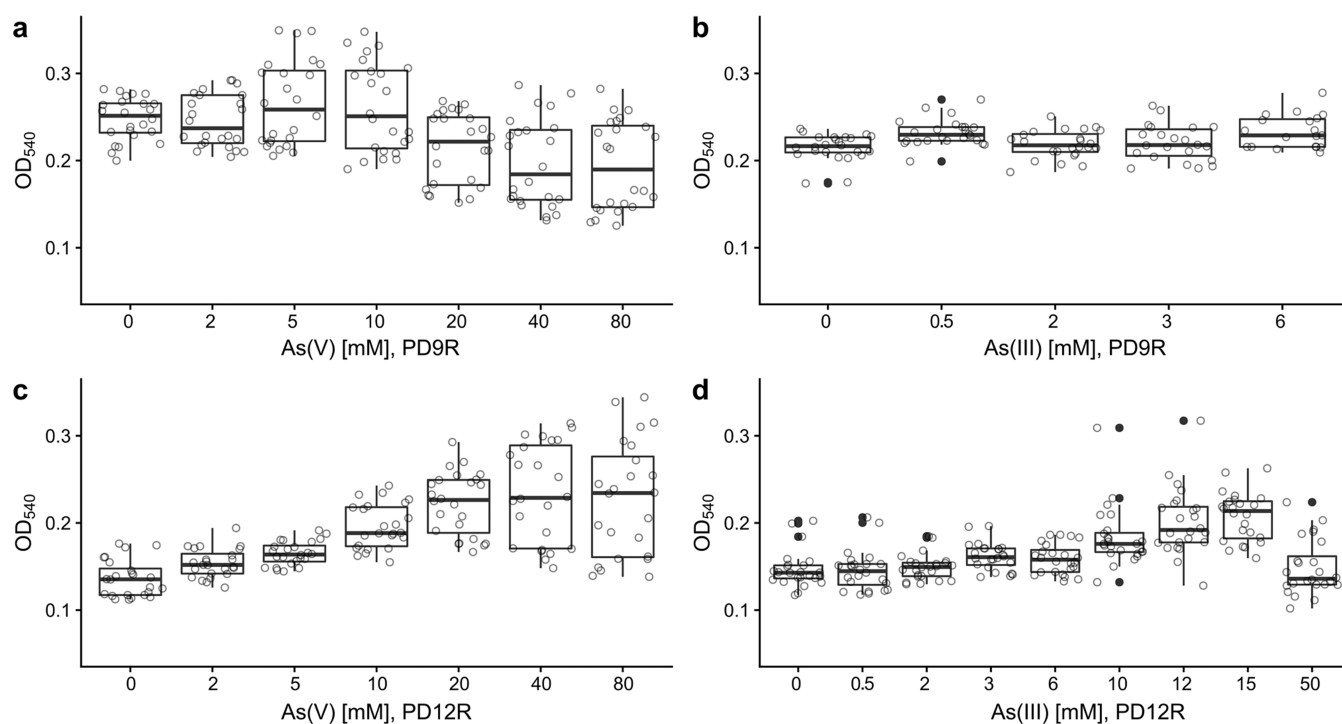


Figure 3. Biofilm formation by PD9R and PD12R in (a, c) 0 to 80 mM As(V), and (b, d) 0 to 50 mM As(III). For PD9R, more than 70% (68/96) of the replicates did not form biofilms above 6 mM As(III) (data omitted from graphs). For PD12R, all replicates formed biofilms at all As(V) and As(III) concentrations tested. One-factor ANOVA ($n = 24$); open dots (○) represent data points used in the construction of box plots (offset for clarity), and closed dots (●) represent data points greater than $1.5 \times \text{IQR}$.

biomass of the PD9R plants was greater by 172% (50 μM) and 216% (100 μM). The PD12R plants had greater mean biomass at 100 μM As(V), an improvement over the 0 and 50 μM As(V) concentrations, where the PD12R plants were 36 and 3% less, respectively.

The observations cannot be clearly attributed to an indirect mechanism (e.g., phytohormone synthesis), a direct mechanism (e.g., biofilm adsorption), or some combination of indirect and direct mechanisms. Nevertheless, in the case of PD9R, the data suggest that the effect of inoculation on root length was independent of As(V) exposure (two-factor ANOVA, PD9R and As(V) interaction, $P = 0.649$), while the interaction between inoculation and As(V) on *A. thaliana* biomass was found to be significant (two-factor ANOVA, $P = 0.032$). Although successful colonization of the *A. thaliana* plants by PD9R and PD12R was not confirmed, it is worth noting that vertical transmission of native seed endophytes has been previously demonstrated in *A. thaliana* and other plants,^{48–50} suggesting that the effects were specific to the arsenic-tolerant endophytes PD9R and PD12R.

Arsenic Induces EPS Synthesis in PD9R and PD12R.

Exposure to As(V) induced EPS synthesis in PD9R and PD12R (Figure 2), a phenomenon that has been widely reported in arsenic-resistant bacteria, including endophytes.^{51–53} At low As(V) concentrations, the mean optical density (OD) of the cell suspensions suggests that EPS synthesis increased along with As(V) concentration in the media for both PD9R and PD12R ($P \ll 0.0001$ and $P = 0.0004$, respectively). In a follow-up experiment conducted in 5–80 mM As(V), both strains had significantly higher levels of EPS synthesis compared to cells cultured without As(V) (one-way ANOVA, PD9R, $P < 0.0005$ and PD12R, $P = 0.021$). However, no differences were found between As(V) treatment

groups above 5 mM As(V) for either strain, suggesting that EPS synthesis reached a maximum threshold at the higher As(V) concentrations (Tukey's HSD, $P > 0.05$). The induction of EPS synthesis by arsenic could explain how arsenic-resistant bacteria escape the toxicity paradox created by extracellular accumulations of the more toxic As(III) expelled from the cell following the cytoplasmic reduction of As(V). To confirm that the OD readings were caused by EPS in the media, and not cell growth, we performed CFU counts on cell suspensions cultured in 0–80 mM As(V), finding that while the CFU counts trended down with increasing As(V) concentration, there were no differences in CFUs between the controls for either strain ($P > 0.05$) (Figure 2c,f).

Differential Responses to Arsenic in PD9R and PD12R Biofilms. Exposure to arsenic reduced PD9R's ability to form biofilms, while PD12R biofilms increased with arsenic concentration (Figure 3). Arsenic concentration had a negative effect on PD9R biofilm formation for both As(V) ($P \gg 0.0001$) and As(III) ($P = 0.038$), with no differences in biofilm formation at the lower concentrations ($P > 0.05$), but declining above 10 mM As(V) and 6 mM As(III), respectively, such that PD9R failed to reliably form biofilms at concentrations greater than 6 mM As(III), with more than 50% of the replicates failing at >6 mM, and 100% failing at >12 mM. Conversely, PD12R biofilms were more resilient than those of PD9R, increasing with arsenic concentration in both As(V) ($P \gg 0.0001$) and As(III) ($P = 0.038$). As with PD9R, the PD12R biofilms in the control group were similar at the lower arsenic concentrations ($P > 0.05$) (Figure 3c,d). However, apart from 50 mM As(III) concentration, the PD12R biofilm formation continued to increase at concentrations greater than 5 mM As(V) and 6 mM As(III), respectively. Further, PD12R formed biofilms in 100% of the replicates in both arsenic species. As

confirmation, the EPS, CFU, and biofilm experiments were repeated on PD12R in a single experiment with As(V). The results were consistent with the prior experiments, where both EPS synthesis ($P < 0.001$) and biofilm formation ($P < 0.001$) increased with As(V) concentration in the media, while CFU did not ($P = 0.921$). The response of biofilm-forming bacteria to arsenic exposure has been shown to vary even among closely related strains. For example, Farasin and colleagues⁵³ observed that among three arsenic-resistant, biofilm-forming *Thiomonas* strains (CB1, CB2, and CB3), only two formed biofilms, while the third responded with increased motility in the presence of arsenite.

Arsenic Adsorption in PD12R Biofilms. Two studies using advanced microscopy have reported the adsorption of arsenic in the biofilms of plant-associated bacteria in vitro, including the endophyte, *Pseudomonas koreensis* AGB-1, and the rhizospheric strains, *Kocuria flava* AB402 and *Bacillus vietnamensis* AB403, although neither study quantified the arsenic accumulations in the biofilms.^{51,52} Given the difference in arsenic resistance and biofilm formation between PD9R and PD12R, we asked whether As(V) was adsorbed by PD12R biofilms, providing a potential mechanism for sequestering As(V) in the host, and whether it was more closely associated with the EPS or cellular fractions. Due to its response during the biofilm experiments, PD9R was excluded.

PD12R biofilms were cultured on sterile polycarbonate membranes placed on MS agar supplemented with 0–5 mM As(V). The membranes, permeable to nutrients and As(V) but not the cells, were used to estimate total biofilm mass and arsenic concentration (see the Detailed Methods section for details). The PD12R biofilms exposed to As(V) were greater in mass than those formed in the absence of As(V) ($P < 0.0001$) but were similar in mass to each other ($P > 0.05$). While the mean arsenic concentration increased in the biofilms with As(V) concentration, when normalized for the original As(V) concentration in the media, the ratios of the arsenic ppm in the biofilms to those in the media were uniform, 6.64 $\times$, 6.28 $\times$, and 6.29 $\times$ respectively, suggesting that the PD12R biofilms were not saturated at these As(V) concentrations.

EPS can be classified according to its association with the cell surface, where the soluble slime EPS consists of biopolymers secreted as colloids, and the insoluble capsular EPS consists of biopolymers attached or integrated into the cell membrane.⁵⁴ For experimental purposes, we defined the slime EPS as soluble (S-EPS) when extracted by centrifugation alone, the capsular EPS as bound (B-EPS) when extracted by centrifugation after incubation with 2% EDTA, and the remaining fraction as the cells. To assess whether arsenic associates more closely with the EPS or cell fractions, PD12R cell suspensions cultured in 2 mM As(V) were separated into the S-EPS, B-EPS, and cell fractions. Exposure to As(V) decreased the mass of the cell fraction by $\sim 50\%$ compared to the controls (26.75 vs 60.27 mg) ($P = 0.007$) (Table 1). However, the reduction in cell mass appeared to be offset by increased EPS synthesis. Despite the reduction in cell mass, the As(V)-treated cells produced 12% more total EPS by mass (sum of S-EPS and B-EPS), primarily as the S-EPS fraction. Approximately 28% of the arsenic was associated with the B-EPS and cell fractions, based on mass balance analysis, with the B-EPS adsorbing twice as much arsenic as the cell fraction (Table 1). Despite the increase in S-EPS, the arsenic concentration in the S-EPS could not be distinguished from the arsenic in the media, and those results have been excluded.

Table 1. (Top) Mean Mass and Arsenic Concentration for PD12R Biofilms Grown on MS Agar with As(V) ($n = 3$)^a

As(V)	mass (mg, dry wt.) $\pm$ Std Dev	As ppm $\pm$ std dev	
control	0.060 $\pm$ 0.02	0.000 $\pm$ 0.00	
0.5 mM	0.198 $\pm$ 0.02	249.1 $\pm$ 14.4	
2 mM	0.171 $\pm$ 0.01	942.7 $\pm$ 65.9	
5 mM	0.167 $\pm$ 0.01	2360.1 $\pm$ 39.9	
fraction	As(V)	mass (mg, dry wt.) $\pm$ std dev	As ppm $\pm$ std dev
cells	control	60.27 $\pm$ 6.6	7.33 $\pm$ 3.5
	2 mM	26.75 $\pm$ 2.5	254.00 $\pm$ 55.5
S-EPS	control	480.07 $\pm$ 43.5	1.00 $\pm$ 1.0
	2 mM	627.77 $\pm$ 221.3	2360.67 $\pm$ 576.0
B-EPS	control	422.77 $\pm$ 7.1	1.33 $\pm$ 1.2
	2 mM	394.50 $\pm$ 6.8	473.33 $\pm$ 69.7

^a(Bottom) Arsenic concentration by biofilm fraction, cells (cellular), S-EPS (soluble EPS), and B-EPS (bound EPS). Arsenic concentration is shown as parts per million (ppm, mg kg⁻¹).

Whole Genome Taxonomic Classification of PD9R and PD12R. PD9R was not matched with any type-strains in the TYGS database and was classified as a potentially novel species. The phylogenetic whole genome analysis clustered PD9R with strains isolated from soils and sediments, including *P. mohnii* DSM 18327 and *P. moorei* DSM 12647, both isolated from polluted sediment (Figure S1a).⁵⁵ Conversely, PD12R closely matched with the type-strain *R. laticis* SAP-17⁵⁶ and clustered with several other plant-associated *Rahnella* isolates (Figure S1b).

Comparative Genomic Survey of Arsenic Resistance Genes in Plant-Associated *Pseudomonas* and *Rahnella* Strains. The arsenic resistance genes in PD9R and PD12R were compared with 42 plant-associated strains, 27 *Pseudomonas* and 15 *Rahnella*, obtained from the NCBI RefSeq database (see the Methods section). The minimum gene set for As(V) resistance is contained within the *arsRBC* operon and includes the transcriptional repressor (ArsR), the As(III) efflux permease (ArsB), and the As(V) reductase (ArsC), although many *ars* operons also include *arsH*, which confers tolerance to trivalent organoarsenicals.⁵⁷ An expanded *ars* operon configuration, *arsRDABC*, includes an ATPase (ArsA) that provides energy to ArsB for the extrusion of As(III), and the dual function ArsD, which is believed to act as an As(III) chaperone for ArsB and a regulator controlling the upper-level expression of the *ars* operon.¹⁸

PD9R carries a robust collection of arsenic resistance genes, including two *ars* operons, *ars1* and *ars2*, with different configurations (Figure 4a). The *ars1* operon has four genes, organized as *arsRBCH*, while the eight-gene *ars2* operon features a three-gene cluster *dsp-gapdh-arsJ*, preceded by two arsenate reductases (*arsC2* and *arsC3*) flanking an *arsR2*, an *acr3* gene, and an *arsH2*. PD9R was the only strain in the plant-associated dataset to carry the *acr3* gene, encoding an As(III) efflux permease first characterized in *Saccharomyces cerevisiae*.²² Interestingly, the *dsp-gapdh-arsJ* gene cluster in PD9R's *ars2* operon contains the only known arsenate efflux protein (ArsJ), first characterized in *P. aeruginosa* DK2.⁵⁸ The genes encode a dual-specific protein phosphatase (DSP) that frequently occurs with *gapdh-arsJ* but whose role is in arsenic tolerance is unknown, GAPDH (glyceraldehyde 3-phosphate dehydrogenase), which catalyzes the formation of an As(V)-phosphoglycerate, and ArsJ permease, an organoarsenical MFS transporter. The *gapdh-arsJ* gene pair was found only in the

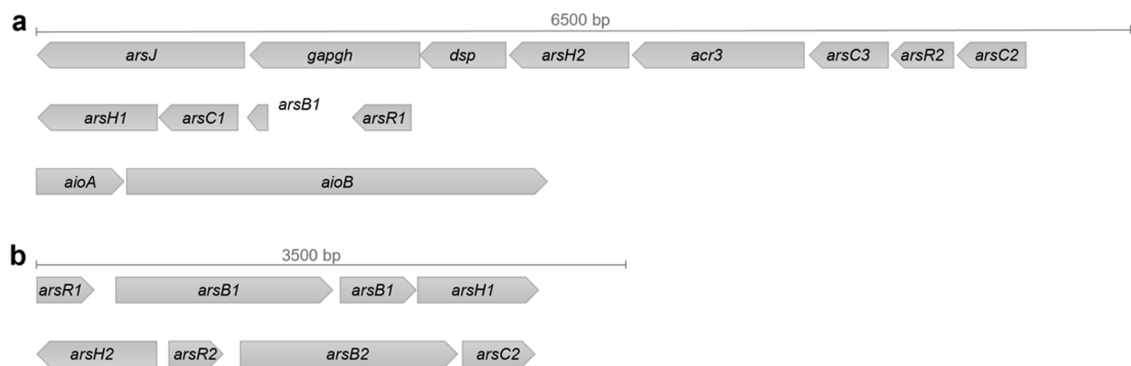


Figure 4. Organization of the operons containing arsenate reductase (Ars) and arsenite oxidase (Aio) genes identified in (a) PD9R and (b) PD12. Note: the *arsB1* gene in PD9R is truncated at 120 bp.

Table 2. PD9R and PD12R Biofilm Genes with KEGG kofamKOALA Ortholog Assignments

strain	pathway/operon	KO product name
<i>pseudomonas</i> sp. PD9R GCA_019200975.1	pel	polysaccharide biosynthesis protein PelA (K21006) PelB (K21007) PelC (K21008) PelD(K21009) PelE(K21010) PelF(K21011) PelG (K21012)
	alginate	alginate O-acetyltransferase complex protein AlgF (K19296) AlgI (K19294) AlgJ (K19295)
		poly(β -D-mannuronate) lyase AlgL [EC:2.4.4.3] (K01729)
		alginate biosynthesis protein AlgX (K19293) AlgK (K19292)
		mannuronan 5-epimerase AlgG [EC:5.1.3.37] (K01795)
		alginate biosynthesis protein AlgK (K19292)
		mannuronan synthase Alg44 [EC:2.4.1.33] (K19290)
		mannuronan synthase Alg8 [EC:2.4.1.33] (K19291)
		cellulose synthase (UDP-forming) [EC:2.4.1.12] (K00694)
<i>R. laticis</i> PD12R GCA_019200965.1	cellulose biosynthesis	cellulose synthase (UDP-forming) [EC:2.4.1.12] (K00694)
		cellulose synthase operon protein B (K20541)
		cellulose synthase operon protein C (K20543)
		cellulose synthase operon protein B (K20541)
		cellulose synthase operon protein C (K20543)
	glycogen	cellulose synthase operon protein B (K20541)
		glucose-1-phosphate adenylyltransferase [EC:2.7.7.27] (K00975)
	colanic acid	starch synthase [EC:2.4.1.21] (K00703)
		glycogen phosphorylase [EC:2.4.1.1] (K00688)
		polysaccharide biosynthesis/export protein (K01991)

485 Pseudomonads, occurring in *Pseudomonas* sp. 401, *Pseudomo-*
486 *nas* sp. Leaf83, and *P. brassicacearum* CDVBN10 in addition to
487 PD9R. By comparison, PD12R has a much simpler arsenic
488 resistance profile, consisting of two *ars* operons possessing the
489 same genes as PD9R's *arsI*, except that the PD12R *arsI*
490 contains a divergently transcribed *arsH* immediately upstream
491 from the *arsR*, *arsH'*RBC, while *ars2* is composed of the more
492 common *arsRBCH* configuration.⁵⁹
493 By comparison, 88% (37/42) of the plant-associated
494 *Pseudomonas* and *Rahnella* strains had at least one *ars* operon,
495 and the *arsRBCH* operon was the most common configuration
496 within and between the genera (Table S4). PD9R was among
497 several of the *Pseudomonas* strains with a second *ars* operon,
498 while PD12R was the only plant-associated *Rahnella* strain
499 with a second *ars* operon. The dual operons could contribute
500 to PD12R's high arsenic tolerance, although it is not clear
501 whether the occurrence of multiple *ars* operons leads to
502 increased arsenate tolerance. For example, of the two studies
503 looking at the dual *ars* operons from *P. putida* KT2440, one
504 found they worked synergistically to account for its high As(V)
505 tolerance,⁶⁰ while the second found that they were differ-
506 entially regulated according to temperature.⁶¹ Only a single
507 plant-associated strain, *R. aquatilis* Ra9-2, carried an operon
508 with the *arsRDABC* configuration.

Among the plant-associated strains surveyed, only PD9R 509
possessed the *aioAB* genes, encoding an As(III) oxidase that 510
catalyzes the periplasmic oxidation of extracellular As(III) to 511
As(V), the less toxic and less mobile of the two arsenic species. 512
While the reaction is typically considered an As(III) 513
detoxification mechanism, some heterotrophs, such as *Agro-* 514
bacterium tumefaciens GW4, and chemolithotrophs, such as 515
Rhizobium sp. NT-26, may also utilize As(III) oxidation for 516
energy generation.⁶² A Megablast search indicated that the 517
PD9R *aioAB* sequence was closely related to *Pseudomonas* 518
isolated from soils and sediments, showing pairwise identities 519
of 91.5% with the *Pseudomonas* strains MPC6 and AO-1, and 520
90.6% with *P. arsenicoxydans* CECT 7543 on >99% coverage 521
(Table S5). 522

Comparative Genomic Survey of EPS Genes in Plant- 523
Associated *Pseudomonas* and *Rahnella* Strains. The in 524
silico analysis of the arsenic resistance mechanisms identified 525
in PD9R and PD12R does not appear to explain the greater 526
observed arsenic tolerance of PD12R in vitro. The EPS 527
synthesis and biofilm formation of the strains vary in response 528
to arsenic. PD12R produces prodigious amounts EPS, but only 529
in the presence of arsenic, and still forms biofilms at high 530
concentrations of As(V) and As(III). Conversely, PD9R 531

synthesizes EPS in the absence of arsenic, and its ability to form biofilms deteriorates as arsenic concentrations increase. PD9R contains the exopolysaccharides of both pellicle synthesis locus (Pel) and alginate (Alg), but not the polysaccharide synthesis locus (Psl), the third exopolysaccharide associated with *P. aeruginosa* biofilms.^{63,64} Nearly all of the plant-associated *Pseudomonas* strains contained loci for Alg, while the Psl and Pel loci were poorly conserved in the plant-associated strains (Table S2). Alginate, the most widely studied *Pseudomonas* exopolysaccharide, is secreted into the surrounding environment by mucoid *Pseudomonas* strains, and the overexpression of alginate increases resistance to antibiotics, heavy metals, and protects the cell against oxidative stress.^{65–67} The alginate overexpression phenotype was not observed in PD9R, which showed consistent growth on agar medium with and without arsenic (data not shown), suggesting that, like other plant-associated *Pseudomonas*, it relies less on alginate in biofilm formation.⁶⁸ The Pel exopolysaccharide is best known for its role in pellicle formation at the air–water interface, but is also involved in cell–cell interactions, structural support in early biofilm formation, and while there is some indication that it may protect against certain antibiotics,⁶⁹ little is known about a specific role in protecting the cells against heavy metals and metalloids (Table 2).

Given the reduced number of *Rahnella* genomes currently available, we conducted a pan-genome analysis to assess whether the biofilm pathway (KEGG map02026) genes involved in bacterial cellulose (Bcs), colanic acid (Wza), and glycogen (Glg) biosynthesis were significantly associated to *Rahnella* strains isolated from plants (Table S5.1). Enrichment analysis results (Table S5.2) show that EPS biosynthetic genes *bcs*, *wza*, and *glg* were commonly shared among the plant-associated and non-plant-associated *Rahnella*, and therefore, not significantly over-represented in any specific group of isolates. In addition, all *Rahnella* strains included in the pan-genome analysis possessed up to three copies of the *bcs*, *wza*, and *glg* genes (Table S5.3), suggesting that a certain degree of genetic and phenotypic plasticity is expected with respect to the colanic acid (slime production) and cellulose (attachment, adherence, and colonization) biosynthesis. The synthesis of colanic acid has been associated with slime-producing members of the *Enterobacteriaceae* family, including *Rahnella*,⁷⁰ and has been linked to the envelope stress response (ESR). The ESR is triggered by an unbalanced redox state, which is triggered upon exposure to toxic compounds such as metals and antibiotics as a consequence of an unbalanced redox state.⁷¹ Therefore, we could speculate that upon exposure to arsenic, the excessive production of S-EPS in PD12R is the result of a general response to arsenic-induced oxidative stress and, as suggested by the pan-genome analysis results,⁷² common to all members of the *Rahnella* genus.

Further experiments will be necessary to determine the role of endophytic arsenic resistance genes and EPS/biofilm formation in plant protection to arsenic-induced stresses, particularly with regard to the in planta formation of biofilms. The in planta experiments demonstrated the potential for arsenic-resistant endophytes to improve host plant growth both in the absence and presence of arsenic-induced stresses. However, it is not possible to say which mechanisms are driving the observed differences without addressing the effect of endophytic PGP on the host resistance mechanisms, in terms of the induction of EPS synthesis and its overproduction by some strains in response to arsenic that has been observed

in a wide range of bacteria in response to antibiotics and metal.^{32,73} This is consistent with our genomic analysis that regardless of the isolation source, the genes involved in EPS synthesis were conserved in multiple copies in the *Rahnella* genomes included in the pan-genome analysis. Mutants with an impaired capacity to form biofilms or synthesize the EPS can be tested to determine whether the plant-associated population, the free-living planktonic cells, or both populations are responsible for the protection of the host to arsenic-induced stresses.

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.est.2c08586>.

Detailed method (File S1); EPA methods 3050b (File S2); JSON script from pan-genome analysis (File S3); TYGS reports for PD12R (File S4) and PD9R (File S5); Murashige and Skoog minimal media recipe (File S6); statistical analyses (File S7); endophyte strains and host plants (Table S1); isolation source of *Rahnella* strains from pan-genome analysis (Table S2); Pct. differences of means for root length and biomass (Table S3); plant-associated strains arsenic and EPS genes (Table S4); Megablast identity matrix for PD9R *aioAB* operon (Table S5); *Rahnella* pan-genome analysis results (Table S5.1); enrichment analysis plant- and non-plant-associated *Rahnella* (Table S5.2); KO assignments *Rahnella* EPS biosynthesis (Table S5.3); WGS assembly statistics for PD12R and PD9R (Table S6); protein accession and KO assignments PD9R and PD12R arsenic genes (Table S7); NCBI Accession numbers for referenced strains (Table S8); PD9R and PD12R WGS phylogenetic trees (Figure S1); and layout of polycarbonate membranes from biofilm experiment (Figure S2) (PDF)

Endophyte strain library, including host plant; strains collected in July 2013, near Pt; defiance (PD) park, tacoma, pierce county, WA (USA) (47° 18' 57.3" N 122° 31' 41.0" W), and near vashon and maury island (VM) marine park, vashon, king county, WA (USA) (47° 22' 44.8" N 122° 24' 19.8" W) (Table S1) (ZIP)

HighRes_Figures_pub (ZIP)

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