Diversity and mechanisms of arsenic resistance among soil bacteria impacted by the ongoing Centralia coal mine fire

Authors:

Taylor K Dunivin[1,2]

Justine Miller[3]

Ashley Shade[1,4]

[1]Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI 48824

[2]Environmental and Integrative Toxicological Sciences Doctoral Program, Michigan State University, East Lansing 48824

[3]Lyman Briggs College, Michigan State University, East Lansing, MI

[4]Program in Ecology, Evolutionary Biology and Behavior, Michigan State University, East Lansing, MI 48824

correspondence: Ashley Shade, shadeash@msu.edu

Originality-Significance Statement. In this work, we examine As resistant bacterial isolates from the surface soils of an underground coal mine fire in Centralia, PA. Characterization of these isolates reveals prevalence of As resistance mechanisms that increase the solubility of arsenic in the environment (arsenate reduction and arsenite extrusion) and evidence for horizontal gene transfer of the gene conferring arsenate reductase, *arsC*. Observations of growth

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phenotype in increasing concentrations suggest modest relationship between fitness in As and taxonomy rather than minimum inhibitory concentration or resistance genotype.

SUMMARY

We examined diversity and mechanisms of microbial arsenic resistance in Centralia, PA, the site of an underground coal seam fire burning since 1962. From hot soil collected from an active vent, we isolated 25 unique arsenic resistant bacteria spanning six genera. Although arsenic concentrations were measured to be relatively low at the time of soil collection, isolates grew with high concentrations of arsenate and arsenite (>300 mM and 20 mM respectively). Among these isolates, we found genes for arsenate reduction and arsenite efflux but not methylation or oxidation. Additionally, we observed evidence for horizontal gene transfer of the arsenate reductase gene *arsC*. Several isolates did not test positive for any of the resistance mechanisms tested, suggesting novelty, untargeted diversity, or nonspecific mechanisms of resistance. Finally, we found that comparisons of isolate growth phenotypes across arsenic concentrations provided insights into cellular responses to arsenic. We suggest that chronic exposures to low arsenic may promote mechanisms that increase environmental solubility and enhance local toxicity (e.g., reduction, arsenite efflux), while intense exposure to arsenic may promote mechanisms that reduce environmental solubility (e.g., oxidation). Thus, disturbance intensity and duration, as well as transferability of the stress response gene(s), together inform microbial community robustness to arsenic and the fate of arsenic in the environment.

INTRODUCTION

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Arsenic (As), a toxic metalloid, is naturally present in the soil, but levels are generally low (<10 ppm) (Han *et al.*, 2003); however, anthropogenic activities, including applying As-containing pesticides, burning fossil fuels, and mining, can greatly increase environmental As concentrations (Wang *et al.*, 2014). Because of the ubiquity of As and its toxicity, biogeochemical cycling of As has important implications for environmental health. The elemental nature of As precludes it from degradation, but its toxicity and mobility can change depending on its oxidation state with arsenate (As^{5+}) being less soluble and less toxic than arsenite (As^{3+}) (Bahar *et al.*, 2013). Bacteria have been shown to oxidize, reduce, methylate, and demethylate As (Mukhopadhyay *et al.*, 2002); thus, environmental bacteria are considered important constituents of As's biogeochemical cycling as the presence and transfer of the genes encoding these activities affect the solubility of As.

As resistance genes are located on chromosomes, plasmids, or both (Andres and Bertin, 2016). Several studies indicate that horizontal gene transfer (HGT) has occurred with As resistance genes (Achour *et al.*, 2007; Cavalca *et al.*, 2010; Villegas-Torres *et al.*, 2011; Jia *et al.*, 2013; Heinrich-Salmeron *et al.*, 2011; Jackson and Dugas, 2003). HGT of the gene encoding arsenate efflux pump (*arsB*) has been seen in environments with low As concentrations (Cai *et al.*, 2009). Stressors aside from As may influence transfer of these resistance genes because HGT rates are expected to increase with disturbance, such as increased temperature and pollution (Aminov, 2011; Williams *et al.*, 1996). Studies that consider compounded stressors may help to determine which genes and corresponding mechanisms are transferred in different environments and thus improve risk assessment and bioremediation.

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Underground coal mine fires act as a long-term disturbance and expose soil microbial communities to increased temperatures as well as coal combustion products. As is naturally present in coal. Coal seam fires are ecologically interesting due to their longevity and effect on environmental chemistry (Janzen and Tobin-Janzen, 2008). Soil surface temperatures above coal mine fire-affected areas range from 21-800°C (Zhang *et al.*, 2013). Steam from these fires emits gases including CO, CO_2, and NH_4 (Melody and Johnston, 2015; Janzen and Tobin-Janzen, 2008; Elick, 2011). In addition to lead, zinc, mercury, and copper, As is found surrounding active vents (steam escaping) of underground coal fires (Pone *et al.*, 2007). The underground coal mine fire in Centralia, PA ignited in 1962 and has been burning ever since. The soil microbial communities overlying the underground fire experience a multitude of fire-related stressors, which may influence resistance phenotypes and their gene transfer. Our overarching objective in this study was to characterize As resistance among bacterial isolates from an active vent in Centralia, PA to assess the diversity of As resistance mechanisms and the diversity of microorganisms harboring these mechanisms. We aimed to gain ecological insights into the mechanisms and inter-species transfer of As resistance, using the Centralia ecosystem as a model stressor. We isolated 25 unique As resistant bacteria, even though chemical analysis revealed comparably low soil As concentrations. Isolate characterization results show novelty and HGT of As resistance genes in Centralia and support the widespread reduction of arsenate in low contamination environments.

RESULTS

Diversity of isolate collection

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As resistant isolates from soil near an active vent of the Centralia coal seam fire spanned six genera, including *Acinetobacter*, *Bacillus*, *Enterobacter*, *Microbacterium*, *Olivibacter*, and *Paenibacillus* (**Fig. 1**; **Table S1**). The colony morphologies of the isolates aligned with expectations from 16S rRNA gene classification, and all isolates showed growth in 24 h at or above 39°C (**Table S1**). This cultivation resulted in an abundance of Firmicutes (48% of isolates).

Molecular characterization of As resistance

As resistance genotypes of the isolates were characterized using endpoint polymerase chain reaction (PCR) with a collection of published primers (**Table S2**) specific for genes conferring resistance via diverse mechanisms, including arsenate reduction, arsenite oxidation, methylation, and arsenite efflux (**Fig. 2A**). Eleven isolates (44%) tested positive for the gene encoding the arsenite efflux pump, *arsB*. Over half of these isolates belong to the genus *Enterobacter*. Three isolates (12%) tested positive for the gene encoding arsenite efflux pump, *ACR3(2)*. Twelve isolates (48%) tested positive for the arsenate reductase gene, *arsC*, and isolate A2727, a *Pseudomonas spp*., tested positive for the gene encoding arsenate respiratory reductase, *arrA*. No isolates tested positive for genes encoding other resistance mechanisms including arsenite oxidase (*aoxB*), arsenite efflux pump (*ACR3(1)*), *or* arsenite methyltransferase (*arsM*). Thus, only mechanisms related to arsenate reduction and arsenite extrusion were found among these Centralia isolates. Notably, four isolates (16%) did not test positive for any As resistance genes tested using published primers, suggesting either undescribed resistance genes or sequence diversity not captured with these primer sets.

Minimum inhibitory concentrations (MIC) and growth phenotypes in As

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115 In parallel to characterization of molecular mechanisms of As resistance, we determined the
116 MICs of arsenate and arsenite for each isolate (**Fig. 2BC**). MIC phenotypes ranged from 50 mM
117 to >300 mM for sodium arsenate and from 3 to 20 mM for sodium arsenite. Both *Pseudomonas*
118 isolates could withstand >300 mM sodium arsenate, which is typical for previously reported
119 pseudomonads resistant to As (Drewniak *et al*., 2008; Das *et*. *al*., 2014). High sodium arsenate
120 resistance (>200 mM) (Jackson *et al*., 2005) was observed in 20% of the isolates. High sodium
121 arsenite resistance (>15 mM) (Drewniak *et al*., 2008) was observed in 16% of the isolates, all of
122 which belong to phylum Firmicutes. Isolates that tested positive for *ACR3(2)* had the highest
123 MICs for sodium arsenate. There were no other observed relationships between genotype and
124 MIC.
125
126 We also analyzed growth phenotypes (lag time, maximum growth rate, and maximum OD_{590}) in
127 As, and our results highlight a relationship between growth in As and taxonomy rather than
128 observed MICs (**Fig. 3; Fig. S1; Fig. S2**). Maximum growth rate (μ) and maximum OD_{590} (A)
129 showed similar patterns in each isolate, so we only report μ here and provide A in supporting
130 materials **(Fig. S2)**. In general, relative growth phenotypes were similar between arsenate and
131 arsenite. Firmicutes maintain basal growth rates in the presence of As. Although *Paenibacillus*
132 had the lowest MICs, isolates from this genus showed the least overall growth phenotype change
133 in As. *Bacillus*, however, exhibit larger increases in lag time (**λ**) compared with *Paenibacillus*.
134 Conversely, the *Olivibacter* isolate showed slight increase in lag time along with more severe
135 reductions in growth rate. Members of *Enterobacter* show large reductions in growth rate as well
136 as increased lag time with increasing As concentrations despite their high MICs. *Pseudomonas*,

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Microbacterium, and *Acinetobacter* have comparatively moderate phenotypes in both arsenate and arsenite.

As transformation

We determined the abilities of isolates to transform arsenate and arsenite using a semiquantitative measure of percent As transformation (Simeonova *et al*., 2004). No isolates oxidized arsenite in this assay (data not shown). By contrast, we observed a wide range of capabilities for arsenate reduction that generally corresponded to isolate taxonomy. Isolates belonging to genera *Olivibacter*, *Paenibacillus*, and *Pseudomonas* did not reduce arsenate (**Fig. 2D**). All isolates belonging to the genus *Enterobacter* had transformation capabilities at or above 50%. Isolates belonging to *Bacillus* had varied arsenate reduction capabilities ranging from 0-90%. The *Microbacterium* isolate (I2748) reduced 10-25% of arsenate in solution, and *Acinetobacter* isolates reduced 0-10% of arsenate. While eight isolates (32%) shown to reduce arsenate *in vitro* tested positive for *arsC*, there were discrepancies between the *in vitro* and molecular data. Sequences of *arsC* from three isolates (A2723, A2733, A2735) encode early stop codons. Five isolates (20%) tested positive for *arsC* but were not shown to reduce arsenate in this assay,; however, the *arsC* sequence in isolate I2747 encodes an early stop codon. Nine isolates (36%) were shown to reduce arsenate but did not test positive for the genes encoding arsenate reductases (*arsC* or *arrA*).

Incongruent phylogenies of arsC and 16S rRNA

Comparing the *arsC* and 16S rRNA phylogenetic trees reveals inconsistencies between gene sequence and phylogeny (**Fig. 4**). All *arsC* sequences from the isolates are most closely related to *arsC* sequences from *Bacillus* strains. Eight isolates spanning three genera (*Bacillus*,

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161 *Paenibacillus*, and *Enterobacter*) had high sequence homology to *Bacillus*-derived *arsC*,
162 suggesting HGT.
163
164 **DISCUSSION**
165 In this study, we describe a collection of As resistant bacteria isolated from the hot soils
166 overlying an underground coal mine fire in Centralia, PA. We later determined that, despite the
167 fire activity at this particular site, the soil had relatively low As concentrations at the time of soil
168 collection (2.58 ppm). Our characterization of these isolates revealed that they had high
169 resistance to both arsenate and arsenite and suggests that arsenate reduction and arsenite
170 extrusion are prevalent among these soil bacteria. We also find that quantifying isolate growth
171 parameters across increasing As concentrations better informs nuanced resistance responses
172 compared with MICs, allowing us to discriminate resistances manifested as differences in lag
173 time to exponential growth, maximum density (OD), or exponential growth rate. Finally,
174 phylogenetic inconsistency of the genes encoding arsenate reductase (*arsC*) in these isolates
175 suggests horizontal gene transfer at the site and the spread of arsenate reduction as a resistance
176 mechanism.
177
178 We observe multiple discrepancies between molecular and functional assays when characterizing
179 the isolates' As resistance. Despite using twelve primer sets to screen for As resistance genes,
180 three isolates with relatively high MICs did not test positive for any As resistance genes screened
181 in this study, highlighting a caveat of using primers for detection that has been reported
182 previously (Achour *et al*., 2007; Cavalca *et al*., 2010). Similarly, we find no correlation between
183 As resistance genotype and MIC in this isolate collection, which was also reported in Achour and
184 colleagues (2007). Furthermore, we observe inconsistencies between molecular results and

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185 arsenate transformation capabilities, suggesting either divergent gene sequences or novel
186 reduction mechanisms (Sarkar *et al*., 2013). We expect that a wider breadth of As resistance gene
187 diversity and novel gene discovery could be captured using cultivation-independent methods,
188 which are our next steps in this line of research.
189
190 We found that growth phenotypes in As provided richer context for resistance than MICs. Our
191 results are consistent with previous reports that Proteobacteria often have high MICs (**Fig. 2B**)
192 (Cavalca *et al*., 2010; Cai *et al*., 2009); however, when simultaneously analyzing reductions in
193 growth with As, our results show distinct growth strategies among lineages, in both arsenate and
194 arsenite (**Fig. 3**). Though other reports have examined growth reduction in the presence of As to
195 find suitable strains for bioremediation (Banerjee *et al*., 2011; Pepi *et al*., 2007), a suite of
196 growth parameters are not typically investigated. Our full characterization of growth in
197 increasing concentrations of As showed a modest relationship between resistance phenotype and
198 taxonomy and highlights discrepancies between fitness in As and MIC. This phylogenetic
199 relationship of growth phenotypes may be attributed to more transient and nonspecific
200 mechanisms of resistance, such as cell envelope permeability to As, oxidative stress response,
201 and regulation of heat shock proteins (Andres and Bertin, 2016; Parvatiyar *et al*., 2005).
202 Accounting for these transient tolerance mechanisms may explain some of the discrepancies
203 between MIC and As resistance genotype (Achour *et al*. 2007) and between MIC and isolate
204 abundance in contaminated sites (Valverde *et al*, 2011). Valverde and colleagues (2011)
205 observed an increase in Firmicutes with increasing As concentrations despite their lower MICs *in*
206 *vitro*. Our findings suggest that Firmicutes, in general, have less extreme growth phenotypes in

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As. Generally, this result questions the accuracy of MICs in predicting the success of an organism in the presence of As.

Microbial arsenate reduction and the transfer of associated functional genes is an important environmental health concern because these processes increase the solubility of environmental As (Bahar *et al.*, 2013). Incongruence between the phylogenetic alignment of *arsC* and the 16S rRNA gene within this isolate collection supports horizontal transfer of genes involved in the reduction of arsenate (**Fig. 4**). Determining the location (chromosomes or plasmids) of and the sequences surrounding these resistance genes would provide insights into mechanisms of transfer. Notably, all *arsC* sequences from this study are most closely related to those of the *Bacillus* lineage, suggesting that the gene originally derived from a *Bacillus*. This suggests that *Bacillus* are contributing to the spread of arsenate reduction in Centralia and may therefore contribute to community robustness against As stressors.

While our isolate collection was not intended to be an exhaustive view of As resistant microorganisms in Centralia, we isolated a relatively large number of As resistant bacteria (25) (**Fig. 1**) as compared to other studies isolating from conditions with low As contamination (<13 ppm) (Jackson *et al.*, 2005; Macur *et al.*, 2004; Achour *et al.*, 2007). This result suggests either an ongoing selection pressure for As resistance in Centralia, potentially due to historical fluctuations in soil As concentrations from the fire, or a general ubiquity of environmental As resistance, which have both been suggested previously (Jackson *et al.*, 2005; Achour *et al.*, 2007, Cavalca *et al.*, 2010). Compounded stressors from the fire may also indirectly increase As resistance. As resistance due to site-specific stressors in Centralia is supported by the sizable number of As resistant isolates of the *Bacillus* lineage rather than *Pseudomonas*, which is

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231 unusual for As resistant isolate collections (Das et al., 2014; Chang *et al.*, 2010; Banerjee *et al.*,
232 2011; Chang *et al.*, 2008; Drewniak *et al.*, 2008; Jackson *et al.*, 2005; Macur *et al.*, 2004; Achour
233 *et al.*, 2007; Wang *et al.*, 2012; Cai *et al.*, 2009). While cultivation bias is present in every study
234 and freezing soil at -80°C prior to cultivation may have influenced our ability to resuscitate some
235 strains (Nelson and Parkinson, 1978), both *Bacillus* and *Pseudomonas* grow using our cultivation
236 conditions (TSA50 at 27°C). The observed low representation of pseudomonads in Centralia is
237 likely due to the high soil temperature, which is outside of their typical growth range (Warth,
238 1978; Tsuji *et al.*,1982). Additionally, a study on bacterial diversity at the site of an underground
239 coal mine fire in China found an abundance of Firmicutes (69.41% of total diversity), especially
240 *Bacillus* and *Paenibacillus* (Zhang *et al.*, 2013). This suggests that Firmicutes may generally be
241 more prominent in coal mine fire-affected soils and contribute to community robustness with
242 their resistance to compounded stressors such as heat and As.
243
244 Our results have important implications for both environmental As mobility and the study of As
245 resistance. We suspect that environmental microbial As resistance is underestimated due to an
246 emphasis on high MICs that are generally above environmental concentrations (Sarkar *et al.*
247 2010), as well as an emphasis on specialized resistance mechanisms rather than generalized
248 stress responses to As. Only genes conferring mechanisms of arsenate reduction and arsenite
249 extrusion were found in Centralia isolates, adding to the growing body of literature reporting an
250 abundance of arsenate reduction genes in sites with low-As concentrations (Cai *et al.*, 2013; Xiao
251 *et al.*, 2016). While arsenite oxidases are generally found in sites with high contamination (Cai *et*
252 *al.* 2009; Sarkar et al. 2013; Drewniak et al. 2012), arsenate reductases and arsenite efflux pumps
253 are generally found in sites with low contamination (Cai *et al.*, 2013; Xiao *et al.*, 2016). For

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254 example, Inskeep and colleagues (2007) repeatedly isolated arsenite oxidase *aroA* in
255 contaminated but not in pristine soils. Also, the presence of *aroA* was greatest in sites with long-
256 term As contamination such as a geothermal spring rather than an orchard with As-containing
257 pesticides (Inskeep *et al*. 2007), suggesting that both intensity and duration of As contamination
258 impact the ratios and abundance of microbial As resistance mechanisms. Thus, microorganisms
259 in As-free soils may respond to new As deposition by transferring available As resistance genes,
260 especially widespread *ars* genes (Cai *et al*., 2013). Therefore, we posit that the environmental
261 fate of As due to microbial metabolism depends on both the intensity and duration of the
262 stressor. If this hypothesis is supported, investigation of chronic and low-exposure environmental
263 As contamination could improve understanding of the microbial ecology of low-exposure sites
264 and inform and bioremediation efforts.
265
266 This case study of As resistance isolates in Centralia suggests a capacity of soil microbial
267 communities for robustness in the face of varied and unanticipated environmental disturbances.
268 When specialized stress responses have potential for transfer to other members, they have the
269 capacity to foster widespread community resistance to particular stressors and to promote overall
270 community robustness. This observation has implications not only for environmental As
271 mobilization and bioremediation but also for mechanisms supporting general microbial
272 community robustness. Thus, the characteristics of the disturbance (intensity and duration of
273 stressor) and the nature of microbial stress response (specialized versus general, and transferable
274 versus fixed) both should be considered to understand the ultimate robustness of a microbial
275 community.
276

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MATERIALS AND METHODS

Soil Collection and Site Description

A soil surface core (20 cm depth and 5.1 cm diameter) was collected in October 2014 from an active vent (steam escaping) in Centralia, Pennsylvania. This vent was selected because it has had historical fire activity since at least 2007 (Elick, 2011) and was the hottest detected at the time of sampling with a measured surface temperature (10 cm depth) of 57.4°C (ambient air temperature was measured to be 13.3°C). Detailed soil geochemical data was assayed by the Michigan State University Soil and Plant Nutrient Laboratory (East Lansing, MI, USA, http://www.spnl.msu.edu/) according to their standard protocols, and total As was measured by Element Materials Technology using the Environmental Protection Agency's method 3050B for sample preparation and ICP-MS (**Table S3)**. Upon sampling, the soil was kept on ice until transport to the lab where it was manually homogenized, sieved through 4 mm mesh, and stored at -80°C until further processing.

Cultivation-dependent soil bacterial community growth

Five grams of soil was removed from -80°C and kept at 4°C for 48 h. The soil was warmed to room temperature for 1 h and then suspended in 25 mL of sterile Dulbecco's phosphate-buffered saline (ThermoFisher; dPBS), vortexed for 2 min, and allowed to settle for 2 min. The supernatant was plated onto 50% tryptic soy agar (Becton Dickinson and Company; TSA50) with 200 μg/mL of cycloheximide added to inhibit fungal growth. Plates were incubated at 27°C for 24 h. To obtain a culture-dependent bacterial community representative of these growth conditions, overgrown plates were scraped to make a 25% glycerol stock and stored at -80°C for future assays.

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301 *Isolation of As Resistant Bacteria*

302 Twenty mL of trypticase soy broth (TSB50) was inoculated with the bacterial community
303 glycerol stock and grown for 6 h with shaking at 200 rpm and 12 mm amplitude. As was not
304 included in the medium to avoid transfer of As resistance genes. The culture was plated onto
305 TSA50 with either 10 mM Na_2HAsO_4 or 1 mM $NaAsO_2$ to screen for arsenate or arsenite
306 resistant colonies, respectively. Ninety-four total colonies (35 from sodium arsenate; 59 from
307 sodium arsenite) were streaked to purity (3x) on their respective media type; 69 pure isolates
308 were recovered and made into 25% glycerol stocks for long term storage at -80°C. From these
309 pure cultures, 25 distinct isolates were identified by genotype with 16S rRNA gene sequencing
310 and by phenotype using MIC assays.
311
312 *Morphological characterization and temperature maxima*
313 Overnight cultures of isolates grown in 3 mL TSB50 were examined using a Nikon E800 Eclipse
314 microscope. Cell morphology was visualized using a photometrics CoolSnap MYO microscope
315 camera (Tuscan, AZ, USA) and Micromanager 4.22 (Edelstein *et al.,* 2014) was used for image
316 acquisition. Cell size was measured using Fiji image analysis software (Schindelin *et al.,* 2012).
317 Colony morphology on TSA50 plates was imaged after incubation at 27°C for 24 h. To measure
318 growth temperature maxima, isolates (2% culture in fresh TSB50) were incubated in a T100
319 Thermo Cycler (BioRad) for 24 h with a thermal gradient (32-52°C). Optical density at 590 nm
320 (OD_{590}) was measured using an Infinite F500 plate reader (Tecan). The maximum temperature
321 for growth was determined as the highest temperature with an increase in OD_{590} from
322 background. This process was repeated for a minimum of two biological replicates per isolate.
323
324 *Endpoint PCR and Amplicon Sequencing*

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Freezer stocks of isolates were inoculated into 3 mL TSB50 and shaken at 27°C at 200 rpm with a 12 mm amplitude until turbid. Genomic DNA (gDNA) was extracted using the E.Z.N.A. Bacterial DNA Kit (Omega Bio-Tek) according to the manufacturer's instructions. Isolated gDNA was quantified with fluorometry using the Qubit dsDNA broad range (BR) assay kit (Invitrogen) and a Qubit 2.0 (Invitrogen) according to the manufacturer's instructions. DNA was stored in sterile Tris-EDTA buffer (Sigma; pH 8) at -20°C.

The near full length 16S rRNA gene was amplified for each isolate using the universal primer pairs Uni-27F and Uni-1492R (**Table S2**). All primers used in this study were desalted and HPLC purified (IDP). PCR amplification of 16S rRNA was carried out in a T100 Thermo Cycler (BioRad) using 25 µL total volume including 30 ng genomic DNA, 0.4 μM of each primer, 0.8 mM dNTPs (Sigma), 2.5 µL 10X Pfu Buffer (Promega), 2X high fidelity Pfu DNA Polymerase (Promega), and nuclease free water to a final volume of 25 μL. The 16S rRNA PCR reaction cycle included a 2 min initial denaturation at 95°C, 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. PCR products were run on a 1% agarose gel for 45 min at 700 mV. The PCR product of 1.4 kb from the 16S rRNA gene was gel extracted using the Wizard SV Gel and PCR Clean Up System (Promega) according to the manufacturer's instructions. Gel extraction products were quantified as before. Purified 16S rRNA amplicons were sequenced using the ABI Prism BigDye Terminator Version 3.1 Cycle sequencing kit by the Michigan State University Genomics Core Research Technology Support Facility (https://rtsf.natsci.msu.edu/genomics/). Forward and reverse 16S rRNA sequences were aligned using CAP3 (v. 3.0, Huang *et al.,* 1999) to obtain near full length 16S rRNA sequences, except for isolates A2707, A2723, and A2735 which could

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not be sequenced using the 1492R primers. For these three isolates, primer U515F (Baker *et al.*, 2003) was used to obtain a near-full length 16S rRNA sequence. Sequences were assigned taxonomy using both the Ribosomal Database Project (RDP) 16S rRNA database (v. 2.10, Wang *et al.*, 2007) and the EzTaxon server (Kim *et al.*, 2012).

Isolates were screened for the following As resistance genes: *arsB*, *ACR3(1)*, *ACR3(2)*, *arsC*, *arrA*, *aoxB*, and *arsM* using published primers that were chosen because of their continued use in the literature (**Table S2**). All PCRs were carried out with published reaction conditions in a T100 Thermo Cycler (BioRad). All *arsC* amplicons were gel extracted and sequenced as described above. At least one forward and one reverse *arsC* sequence was merged in CodonCodeAligner (v. 6.0.2, Codon Code Corporation) to create *arsC* contigs.

Phylogenetic Analysis

To compare the 16S rRNA phylogenetic diversity of Centralia As resistant isolates to previous reports, existing literature was used to curate 55 16S rRNA sequences (> 700 bps) from other studies that also confirmed their As resistance in the isolates by selection on As-containing media. Closest 16S rRNA gene relatives deposited at the NCBI (http://www.ncbi.nlm.nih.gov/) were also included in the analysis. Sequences were aligned using the RDP aligner (Cole *et al.*, 2014). RDP characters were removed from aligned sequences using BioEdit (v. 7.2.5, Hall, 1999). All trees were made with MEGA7.0 (Kumar *et al.*, 2016) and constructed with the Neighbor-joining algorithm using the Kimura 2 parameter model with 1000 bootstrap replications.

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To examine the phylogeny of *arsC* sequences, sequences of *arsC* from the isolates were compared with related chromosomal *arsC* sequences deposited at the NCBI along with their corresponding 16S rRNA gene sequences. The *arsC* sequences from 16S rRNA relatives were found by searching chromosomes deposited at the NCBI, and closest NCBI matches for *arsC* were determined using BLAST. A corresponding 16S rRNA tree was made using *arsC* sequences from the isolates and their phylogenetic relatives.

MICs of Arsenate and Arsenite

To determine the MICs of arsenate and arsenite as well as their growth phenotypes, isolates were inoculated from 25% glycerol stocks into 3 mL TSB50 and incubated with shaking at 200 rpm with a 12 mm amplitude at 27°C for 6 h. Inocula were added to a 96-well plate with As-containing TSB50 to make a 1% solution. Concentrations tested include 0, 10, 50, 100, 150, 200, 250, and 300 mM sodium arsenate and 0, 1, 3, 5, 7, 10, 14, and 20 mM sodium arsenite. Plates were shaken continuously at 288 rpm with a 3 mm amplitude in an Infinite500 plate reader (Tecan) for 72 h at 27 ± 1°C. OD_{590} was measured every 15 min. Growth experiments were repeated with at least two biological replicates for each isolate, and growth curves for further analysis were made using technical triplicates.

The R environment for statistical computing (R Core Development Team, 2015) was used to plot growth curves and analyze key features of growth inhibition across the range of arsenate and arsenite concentrations tested using a modified script (http://bconnelly.net/2014/04/analyzing-microbial-growth-with-r/). Using the GroFit package (Kahm *et al.*, 2010), splining was used to extract growth parameters including time to exponential growth, maximum growth rate, and

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maximum OD_{590}. When splining was not appropriate (e.g. curves do not have a smooth fit), parameters were estimated parametrically using either Logistic, Gompertz, or Richards models informed by their Akaike information criterion (AIC) (Akaike, 1973). Parameters for each isolate in TSB50 containing As were normalized to As-free controls. All R scripts are freely available on GitHub (https://github.com/ShadeLab/Arsenic_Growth_Analysis/tree/master/R_scripts).

As Transformation Capabilities

The ability of the isolates to reduce arsenate or oxidize arsenite was measured using a modified silver nitrate colorimetric assay as described previously (Simeonova *et al.,* 2004). 0.1 M Tris-HCl (pH 7.3) was used as a reaction buffer instead of 0.2 M, and 1.33 mM sodium arsenate or sodium arsenite was used instead of 0.67 mM. Cells were inoculated in 3 mL TSB50 and incubated at 27°C for 15 h before plating. Cells were washed as indicated in Simeonova *et al.* (2004), and 20 μL of the washed cell suspension was incubated with 80 μL of 0.1 M Tris-HCl and 1.33 mM for 72 h at 27°C. Two standard curves with different ratios of sodium arsenate and sodium arsenite (0:1, 1:10, 1:4, 1:1) were also included alongside the cells. Cell viability was tested by patching onto fresh TSA50 plates after 72 h. After the silver nitrate reaction was initiated, plate photographs were taken, and colorimetric changes were assessed. This protocol was performed with at least two biological replicates plated in duplicate.

Table and Figure legends

Figure 1. Phylogenetic tree of 16S rRNA sequences from Centralia As resistant isolates. Isolates from this study were compared with isolates from other studies that cultivated As resistant isolates from soil. **A)** Actinobacteria, Proteobacteria, and Sphingobacteria. Scale bar of 0.05

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indicates a 5% difference in nucleotide sequence. **B)** Firmicutes. Scale bar of 0.01 indicates a 1% difference in nucleotide sequence.

Figure 2. As resistance genotypes and phenotypes of isolated bacterial strains. **A)** Presence of As resistance genes from end-point PCR are indicated (+). **B)** MICs of sodium arsenate and arsenite. **C)** Percent range arsenate reduced.

Figure 3. Growth phenotypes of isolates in increasing concentrations of As. Lag time (λ) and maximum growth rate (μ) of isolates in TSB50 with increasing concentrations of **A)** arsenate and **B)** arsenite normalized to growth without As.

Figure 4. Comparison of *arsC* and 16S rRNA gene sequences from As resistant isolates. **A)** A neighbor joining tree of 16S rRNA gene sequences. Scale bar of 0.02 indicates a 2% difference in nucleotide sequence. **B)** A neighbor joining tree of corresponding *arsC* sequences from isolates from this study. Scale bar of 0.01 indicates a 1% difference in nucleotide sequence. Color indicates isolate genus. Bootstrap values greater than 50% are indicated at the corresponding node, and color refers to genus.

Accession numbers

16S rRNA and *arsC* sequences can be found in NCBI with the following accession numbers: KX825887-KX825911 for 16S rRNA and ###-### for *arsC*.

Acknowledgements

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References

Achour, A. R., Bauda, P., Billard, P. (2007). Diversity of arsenite transporter genes from arsenic-resistant soil bacteria. *Res Microbiol*, *158*(2), 128–137.

Akaike H. (1973). Information Theory and an Extension of the Maximum Likelihood Principle. In B. N. Petrov, F. Csaki (eds.), *Second International Symposium on Information Theory*, pp. 267-281. Akademiai Kiado.

Aminov, R. I. (2011). Horizontal gene exchange in environmental microbiota. *Front Microbiol*, *2*(July), 1–19.

Andres, J., and Bertin, P. N. (2016). The microbial genomics of arsenic. *FEMS Microbiol Rev*, (July 2015), fuv050.

Bahar, M. M., Megharaj, M., Naidu, R. (2013). Bioremediation of arsenic-contaminated water: Recent advances and future prospects. *Water Air Soil Poll*, *224*(12), 1–20.

Banerjee, S., Datta, S., Chattyopadhyay, D., Sarkar, P. (2011). Arsenic accumulating and transforming bacteria isolated from contaminated soil for potential use in bioremediation. *J Environ Sci Heal A*, *46*(November 2014), 1736–47.

Cai, L., Liu, G., Rensing, C., Wang, G. (2009). Genes involved in arsenic transformation and resistance associated with different levels of arsenic-contaminated soils. *BMC Microbiol*, *9*, 4.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2451v1 | CC BY 4.0 Open Access | rec: 15 Sep 2016, publ: 15 Sep 2016

463 Cai, L., Yu, K., Yang, Y., Chen, B. W., Li, X. D., Zhang, T. (2013). Metagenomic exploration
464 reveals high levels of microbial arsenic metabolism genes in activated sludge and coastal
465 sediments. *Appl Microbiol Biot*, *97*(21), 9579–9588.
466 Cavalca, L., Zanchi, R., Corsini, A., Colombo, M., Romagnoli, C., Canzi, E., *et al*. (2010).
467 Arsenic-resistant bacteria associated with roots of the wild Cirsium arvense (L.) plant from
468 an arsenic polluted soil, and screening of potential plant growth-promoting characteristics.
469 *Syst Appl Microbiol*, *33*, 154–164.
470 Chang, J.-S., Kim, Y.-H., Kim, K.-W. (2008). The ars genotype characterization of arsenic-
471 resistant bacteria from arsenic-contaminated gold-silver mines in the Republic of Korea.
472 *Appl Microbiol Biot*, *80*(1), 155–65.
473 Chang, J. S., Yoon, I. H., Lee, J. H., Kim, K. R., An, J., Kim, K. W. (2010). Arsenic
474 detoxification potential of aox genes in arsenite-oxidizing bacteria isolated from natural and
475 constructed wetlands in the Republic of Korea. *Environ Geochem Hlth*, *32*(2), 95–105.
476 Cole, J. R., Wang, Q., Fish, J. A., Chai, B., McGarrell, D. M., Sun, Y., *et al*. (2014). Ribosomal
477 Database Project: Data and tools for high throughput rRNA analysis. *Nucleic Acids Res*,
478 *42*(D1), 633–642.
479 Das, S., Jean, J. S., Kar, S., Chou, M. L., Chen, C. Y. (2014). Screening of plant growth-
480 promoting traits in arsenic-resistant bacteria isolated from agricultural soil and their
481 potential implication for arsenic bioremediation. *J Hazard Mater*, *272*, 112–120.
482 Drewniak, L., Styczek, A., Majder-Lopatka, M., Sklodowska, A. (2008). Bacteria, hypertolerant
483 to arsenic in the rocks of an ancient gold mine, and their potential role in dissemination of
484 arsenic pollution. *Environ Pollut*, *156*(3), 1069–1074.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2451v1 | CC BY 4.0 Open Access | rec: 15 Sep 2016, publ: 15 Sep 2016

485 Drewniak, L., Maryan, N., Lewandowski, W., Kaczanowski, S., & Sklodowska, A. (2012). The
486 contribution of microbial mats to the arsenic geochemistry of an ancient gold mine.
487 *Environmental Pollution*, *162*, 190–201. http://doi.org/10.1016/j.envpol.2011.11.023
488 Edelstein, A. D., Tsuchida, M. A., Amodaj, N., Pinkard, H., Vale, R. D., Stuurman, N. (2014).
489 Advanced methods of microscope control using μManager software. *J Biol Methods*, *1*(2),
490 e10.
491 Elick, J. M. (2011). Mapping the coal fire at Centralia, Pa using thermal infrared imagery. *Int J*
492 *Coal Geol*, *87*(3-4), 197–203.
493 Hall, T. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis
494 program for Windows 95/98/NT. *Nucl Acid S*.
495 Han, F. X., Su, Y., Monts, D. L., Plodinec, M. J., Banin, A., Triplett, G. E. (2003). Assessment
496 of global industrial-age anthropogenic arsenic contamination. *Naturwissenschaften*, *90*(9),
497 395–401.
498 Heinrich-Salmeron, A., Cordi, A., Brochier-Armanet, C., Halter, D., Pagnout, C., Abbaszadeh-
499 Fard, E., *et al*. (2011). Unsuspected diversity of arsenite-oxidizing bacteria as revealed by
500 widespread distribution of the aoxB Gene in prokaryotes. *Appl Environ Microb*, *77*(13),
501 4685–4692.
502 Huang, X., Madan, a. (1999). CAP 3: A DNA sequence assembly program. *Genome Res*, *9*(906),
503 868–877.
504 Jackson, C. R., Dugas, S. L. (2003). Phylogenetic analysis of bacterial and archaeal arsC gene
505 sequences suggests an ancient, common origin for arsenate reductase. *BMC Evol Biol*, *3*, 18.
506 Jackson, C. R., Dugas, S. L., Harrison, K. G. (2005). Enumeration and characterization of
507 arsenate-resistant bacteria in arsenic free soils. *Soil Biol Biochem*, *37*(12), 2319–2322.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2451v1 | CC BY 4.0 Open Access | rec: 15 Sep 2016, publ: 15 Sep 2016

508 Jia, Y., Huang, H., Zhong, M., Wang, F., Zhang, L., Zhu, Y.-G. (2013). Microbial arsenic
509 methylation in soil and rice rhizosphere-Support Information. *Environ Sci Technol*, *47*(7),
510 3141–3148.
511 Kahm, M., Hasenbrink, G., Lichtenberg-frate, H., Ludwig, J., Kschischo, M. (2010). Grofit:
512 Fitting biological growth curves. *J Stat Softw*, *33*(7), 1–21.
513 Kim, O. S., Cho, Y. J., Lee, K., Yoon, S. H., Kim, M., Na, H., *et al*. (2012). Introducing
514 EzTaxon-e: A prokaryotic 16s rRNA gene sequence database with phylotypes that represent
515 uncultured species. *Int J Syst Evol Micr*, *62*(PART 3), 716–721.
516 Kumar, S., Stecher, G., Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis
517 version 7.0 for bigger datasets. *Mol Biol Evol*, msw054.
518 Macur, R.E., Jackson, C.R., Botero, L.M., McDermott, T.R., Inskeep, W.P. (2004). Bacterial
519 populations associated with the oxidation and reduction of arsenic in an unsaturated soil.
520 *Environ Sci Technol*, vol. 38, pp. 104-111, *38*(1), 104–111.
521 Melody, S. M., Johnston, F. H. (2015). Coal mine fires and human health: What do we know? *Int*
522 *J Coal Geol*, *152, Part*, 1–14.
523 Mukhopadhyay, R., Rosen, B. P., Phung, L. T., Silver, S. (2002). Microbial arsenic: From
524 geocycles to genes and enzymes. *FEMS Microbiol Rev*, *26*(3), 311–325.
525 Nelson, L. M., Parkinson, D. (1978). Effect of freezing and thawing on survival of three bacterial
526 isolates from an arctic soil. *Can J Microbiol*, *24*(12), 1468–74.
527 Pepi, M., Volterrani, M., Renzi, M., Marvasi, M., Gasperini, S., Franchi, E., Focardi, S. E.
528 (2007). Arsenic-resistant bacteria isolated from contaminated sediments of the Orbetello
529 Lagoon, Italy, and their characterization. *J Appl Microbiol*, *103*(6), 2299–2308.
530 Pone, J. D. N., Hein, K. A. A., Stracher, G. B., Annegarn, H. J., Finkleman, R. B., Blake, D. R.,

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2451v1 | CC BY 4.0 Open Access | rec: 15 Sep 2016, publ: 15 Sep 2016

et al. (2007). The spontaneous combustion of coal and its by-products in the Witbank and Sasolburg coalfields of South Africa. *Int J Coal Geol*, *72*(2), 124–140.

Quemeneur, M., Heinrich-Salmeron, A., Muller, D., Lievremont, D., Jauzein, M., Bertin, P. N., *et al*. (2008). Diversity Surveys and Evolutionary Relationships of aoxB Genes in Aerobic Arsenite-Oxidizing Bacteria. *Appl Environ Microb*, *74*(14), 4567–4573.

Sarkar, A., Kazy, S. K., Sar, P. (2013). Characterization of arsenic resistant bacteria from arsenic rich groundwater of West Bengal, India. *Ecotoxicology*, *22*, 363–376.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., *et al*. (2012). Fiji: an open-source platform for biological-image analysis. *Nat Meth*, *9*(7), 676–682.

Simeonova, D. D., Lièvremont, D., Lagarde, F., Muller, D. A. E., Groudeva, V. I., Lett, M. C. (2004). Microplate screening assay for the detection of arsenite-oxidizing and arsenate-reducing bacteria. *FEMS Microbiol Lett*, *237*(2), 249–253.

Song, B., Chyun, E., Jaffé, P. R., Ward, B. B. (2009). Molecular methods to detect and monitor dissimilatory arsenate-respiring bacteria (DARB) in sediments. *FEMS Microbiol Ecol*, *68*(1), 108–17.

Sun, Y., Polishchuk, E. A., Radoja, U., Cullen, W. R. (2004). Identification and quantification of arsC genes in environmental samples by using real-time PCR. *J Microbiol Meth*, *58*, 335–349.

R Core Development Team (2015). R: A language and environment for statistical computing [Internet]. Vienna, Austria: R Foundation for Statistical Computing; 2013. *Document freely available on the internet at: http://www. r-project. Org*.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2451v1 | CC BY 4.0 Open Access | rec: 15 Sep 2016, publ: 15 Sep 2016

553 Tsuji, A., Kaneko, Y., Takahashi, K., Ogawa, M., Goto, S. (1982). The effects of temperature
554 and pH on the growth of eight enteric and nine glucose non-fermenting species of gram-
555 negative rods. *Microbiol Immunol*, *26*(1), 15–24.
556 Valverde, A., Gonzalez-Tirante, M., Medina-Sierra, M., Santa-Regina, I., Garcia-Sanchez, A.,
557 Igual, J. M. (2011). Diversity and community structure of culturable arsenic-resistant
558 bacteria across a soil arsenic gradient at an abandoned tungsten-tin mining area.
559 *Chemosphere*, *85*(1), 129–134.
560 Villegas-Torres, M. F., Bedoya-Reina, O. C., Salazar, C., Vives-Florez, M. J., Dussan, J. (2011).
561 Horizontal arsC gene transfer among microorganisms isolated from arsenic polluted soil. *Int*
562 *Biodeter Biodegr*, *65*(1), 147–152.
563 Wang, Q., Garrity, G. M., Tiedje, J. M., Cole, J. R. (2007). Naïve Bayesian classifier for rapid
564 assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microb*,
565 *73*(16), 5261–5267.
566 Wang, X., Rathinasabapathi, B., Oliveira, L. M. De, Guilherme, L. R. G., Ma, L. Q. (2012).
567 Bacteria-mediated arsenic oxidation and reduction in the growth media of arsenic
568 hyperaccumulator Pteris vittata. *Environ Sci Technol*, *46*(20), 11259–11266.
569 Wang, P., Sun, G., Jia, Y., Meharg, A. A., Zhu, Y. (2014). A review on completing arsenic
570 biogeochemical cycle: Microbial volatilization of arsines in environment. *J Environ Sci-*
571 *China*, *26*(2), 371–381.
572 Warth, A. D. (1978). Relationship between the heat resistance of spores and the optimum and
573 maximum growth temperatures of Bacillus species. *J Bacteriol*, *134*(3), 699–705.
574 Williams, H. G., Day, M. J., Fry, J. C., Stewart, G. J. (1996). Natural transformation in river
575 epilithion. *Appl Environ Microbiol*, *62*(8), 2994–2998.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.2451v1 | CC BY 4.0 Open Access | rec: 15 Sep 2016, publ: 15 Sep 2016

576 Xiao, K.-Q., Li, L.-G., Ma, L.-P., Zhang, S.-Y., Bao, P., Zhang, T., Zhu, Y.-G. (2016).
577 Metagenomic analysis revealed highly diverse microbial arsenic metabolism genes in paddy
578 soils with low-arsenic contents. *Environ Pollut*, *211*, 1–8.
579 Zhang, T., Xu, J., Zeng, J., Lou, K. (2013). Diversity of prokaryotes associated with soils around
580 coal-fire gas vents in MaNasi county of Xinjiang, China. *A Van Leeuw Int J G*, *103*(1),
581 23–36.
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Figure 1

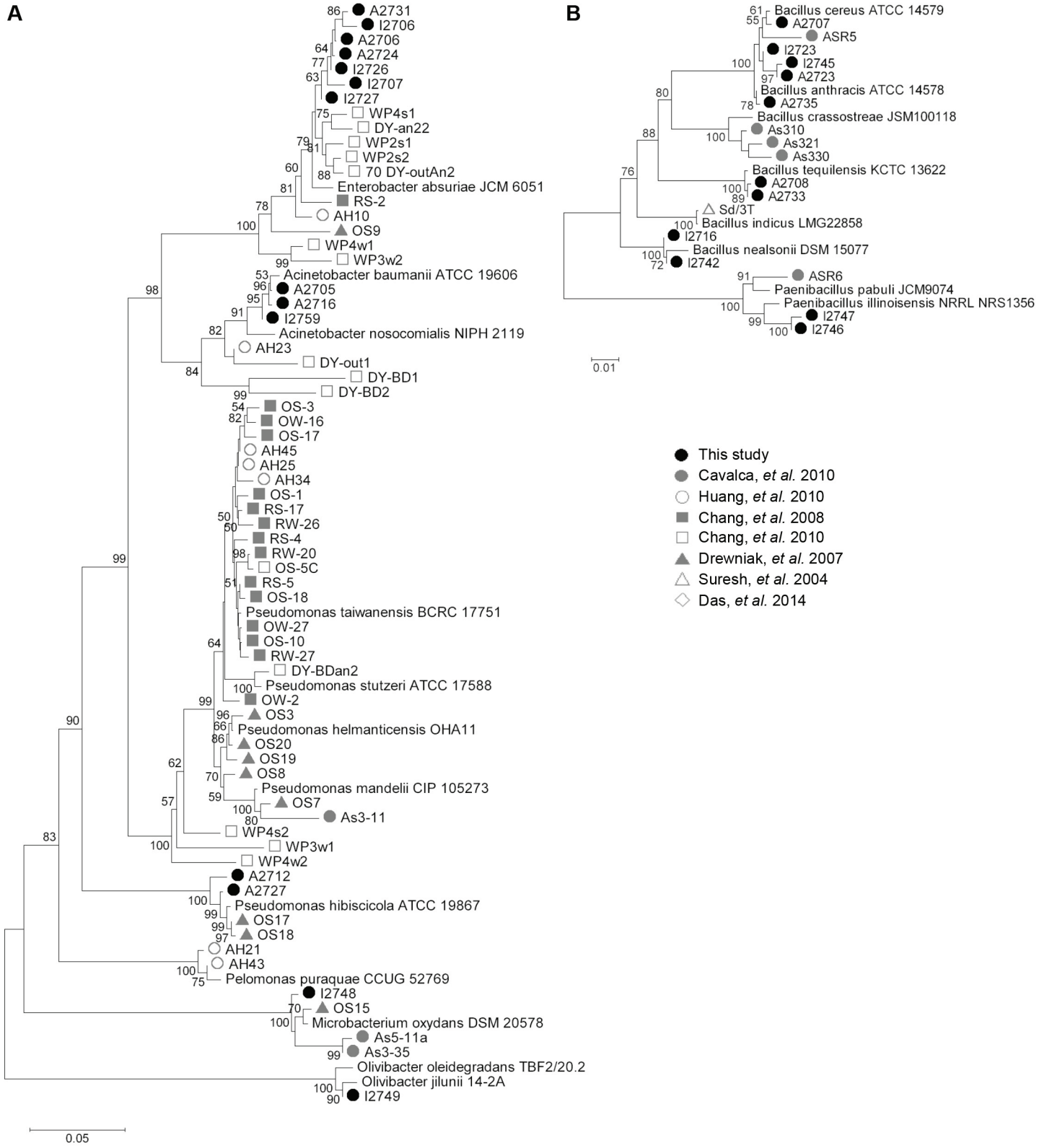

A
A2731
I2706
A2706
A2724
I2726
I2707
I2727
WP4s1
DY-an22
WP2s1
WP2s2
70 DY-outAn2
Enterobacter absuriae JCM 6051
RS-2
AH10
OS9
WP4w1
WP3w2
Acinetobacter baumanii ATCC 19606
A2705
A2716
I2759
Acinetobacter nosocomialis NIPH 2119
AH23
DY-out1
DY-BD1
DY-BD2
OS-3
OW-16
OS-17
AH45
AH25
AH34
OS-1
RS-17
RW-26
RS-4
RW-20
OS-5C
RS-5
OS-18
Pseudomonas taiwanensis BCRC 17751
OW-27
OS-10
RW-27
DY-BDan2
Pseudomonas stutzeri ATCC 17588
OW-2
OS3
Pseudomonas helmanticensis OHA11
OS20
OS19
OS8
Pseudomonas mandelii CIP 105273
OS7
As3-11
WP4s2
WP3w1
WP4w2
A2712
A2727
Pseudomonas hibiscicola ATCC 19867
OS17
OS18
AH21
AH43
Pelomonas puraquae CCUG 52769
I2748
OS15
Microbacterium oxydans DSM 20578
As5-11a
As3-35
Olivibacter oleidegradans TBF2/20.2
Olivibacter jilunii 14-2A
I2749
0.05
B
Bacillus cereus ATCC 14579
A2707
ASR5
I2723
I2745
A2723
Bacillus anthracis ATCC 14578
A2735
Bacillus crassostreae JSM100118
As310
As321
As330
Bacillus tequilensis KCTC 13622
A2708
A2733
Sd/3T
Bacillus indicus LMG22858
I2716
Bacillus nealsonii DSM 15077
I2742
ASR6
Paenibacillus pabuli JCM9074
Paenibacillus illinoisensis NRRL NRS1356
I2747
I2746
0.01
This study
Cavalca, et al. 2010
Huang, et al. 2010
Chang, et al. 2008
Chang, et al. 2010
Drewniak, et al. 2007
Suresh, et al. 2004
Das, et al. 2014

Figure 2

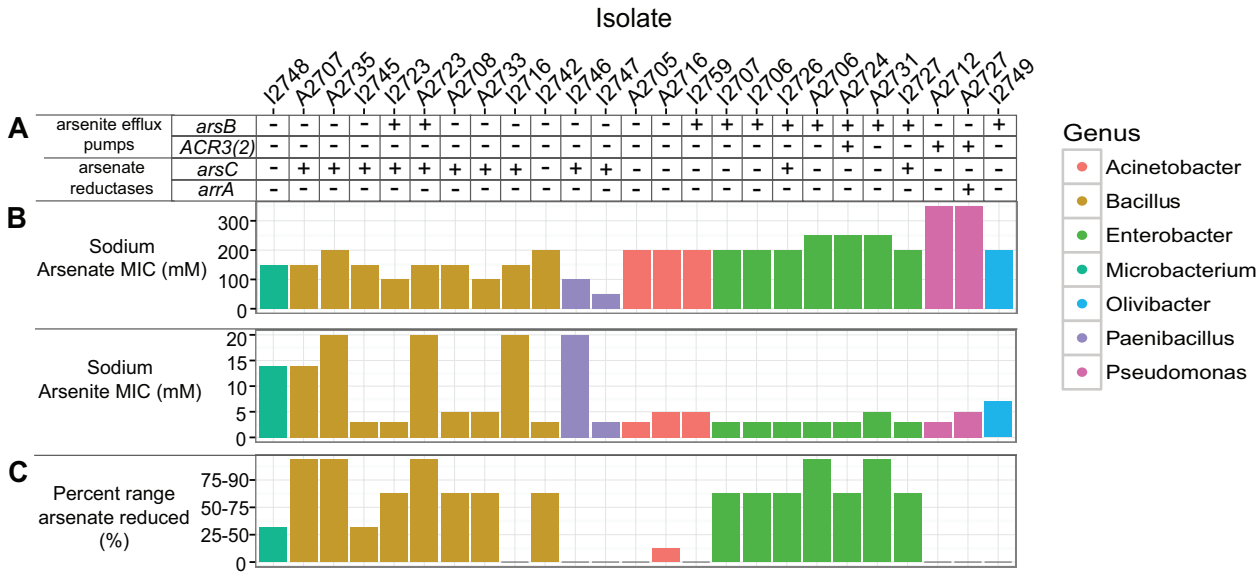

Isolate
I2748
A2707
A2735
I2745
I2723
A2723
A2708
A2733
I2716
I2742
I2746
I2747
A2705
A2716
I2759
I2707
I2706
I2726
A2706
A2724
A2731
I2727
A2712
A2727
I2749
A
arsenite efflux pumps
arsB
ACR3(2)
arsenate reductases
arsC
arrA
B
Sodium Arsenate MIC (mM)
300
200
100
0
Sodium Arsenite MIC (mM)
20
15
10
5
0
C
Percent range arsenate reduced (%)
75-90
50-75
25-50
0
Genus
Acinetobacter
Bacillus
Enterobacter
Microbacterium
Olivibacter
Paenibacillus
Pseudomonas

Figure 3

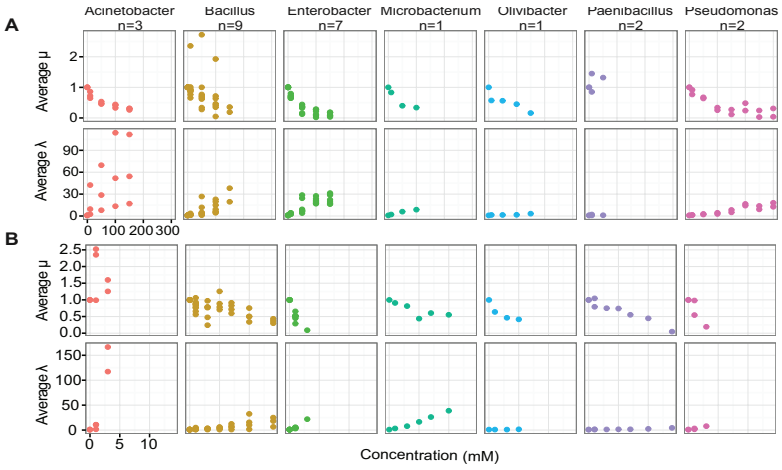
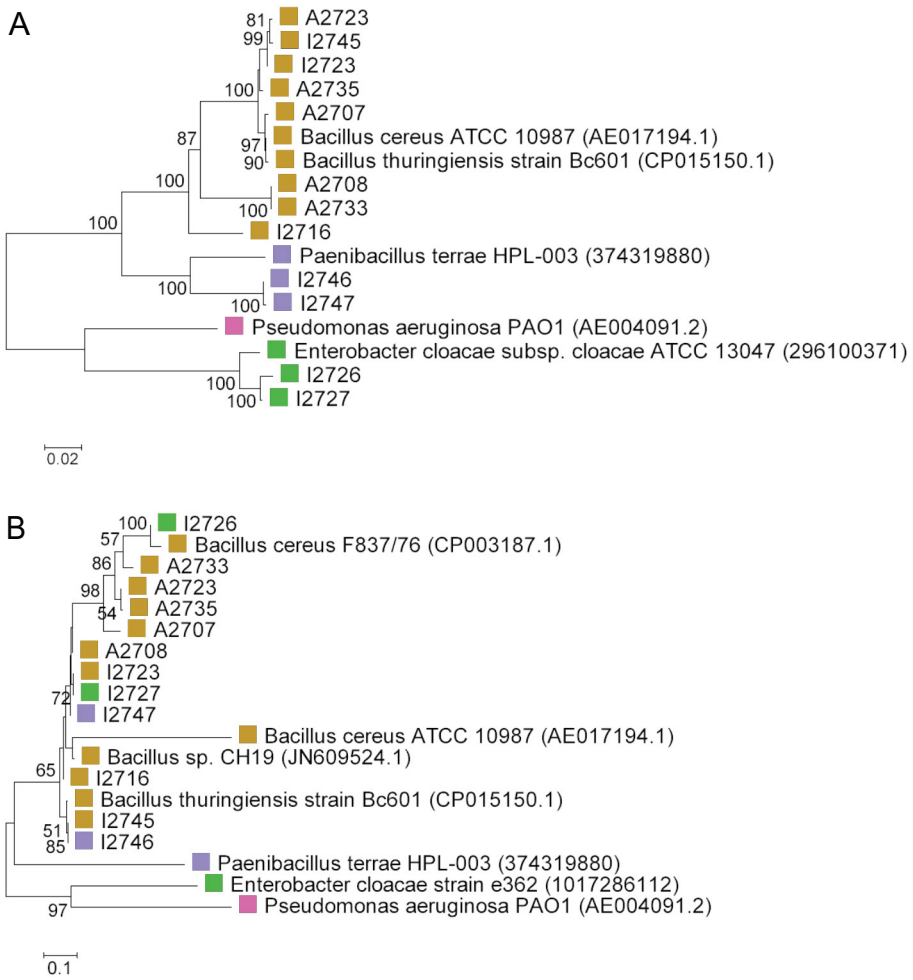


Figure 4


A
A2723
I2745
I2723
A2735
A2707
Bacillus cereus ATCC 10987 (AE017194.1)
Bacillus thuringiensis strain Bc601 (CP015150.1)
A2708
A2733
I2716
Paenibacillus terrae HPL-003 (374319880)
I2746
I2747
Pseudomonas aeruginosa PAO1 (AE004091.2)
Enterobacter cloacae subsp. cloacae ATCC 13047 (296100371)
I2726
I2727
0.02
B
I2726
Bacillus cereus F837/76 (CP003187.1)
A2733
A2723
A2735
A2707
A2708
I2723
I2727
I2747
Bacillus cereus ATCC 10987 (AE017194.1)
Bacillus sp. CH19 (JN609524.1)
I2716
Bacillus thuringiensis strain Bc601 (CP015150.1)
I2745
I2746
Paenibacillus terrae HPL-003 (374319880)
Enterobacter cloacae strain e362 (1017286112)
Pseudomonas aeruginosa PAO1 (AE004091.2)
0.1

Supplementary Table 1. Phenotypes of arsenic resistant isolates.

Isolate	Closest 16S rRNA gene sequence described (% similarity)	Colony Morphology	Temperature Maximum (°C)	Length (µm)	Width (µm)
I2706	*Enterobacter absuriae JM 6051* (99.43%)		44.3	1.43	1.21
I2707	*Enterobacter absuriae JM 6051* (99.35%)		44.3	1.34	1.16
I2716	*Bacillus nealsonii DSM 150-7577* (99.49%)		44.3	4.63	1.16
I2723	*Bacillus anthrasis ATT 14578* (100%)		44.3	4.46	1.36
I2726	*Enterobacter absuriae JM 6051* (99.5%)		44.3	2.37	1.45
I2727	*Enterobacter absuriae JM 6051* (99.56%)		44.3	2.89	1.05
I2742	*Bacillus nealsonii DSM 150-7577* (99.49%)		44.3	2.60	0.85
I2745	*Bacillus anthrasis ATT 14578* (99.86%)		44.3	4.11	0.90
I2746	*Paenibacillus xylanilytius XIL14* (98.63%)		44.3	1.68	1.46
I2747	*Paenibacillus xylanilytius XIL14* (98.58%)		39.7	3.96	1.12
I2748	*Mirobaterium paraoxydans F36* (99.85%)		47.7	1.19	1.14
I2749	*Olivibater oleidegrans TBF2/20.2* (99.42%)		44.3	1.58	1.23
I2759	*Acinetobacter baumanii AT 19606* (99.78%)		44.3	1.20	1.16
A2705	*Acinetobacter baumanii AT 19606* (99.64%)		44.3	1.25	1.19
A2706	*Enterobacter absuriae JM 6051* (99.50%)		44.3	2.07	1.10
A2707	*Bacillus anthrasis ATT 14578* (100%)		44.3	3.83	0.91
A2708	*Bacillus subtilis subsp. inoquosorum KT 13429* (99.93%)		52.0	3.26	0.90
A2712	*Pseudomonas hibisicola AT 19867* (99.36%)		39.7	2.01	1.01
A2716	*Acinetobacter baumanii AT 19606* (99.78%)		44.3	1.12	0.96
A2723	*Bacillus anthrasis ATT 14578* (99.73%)		44.3	3.20	1.64
A2724	*Enterobacter absuriae JM 6051* (99.57%)		44.3	1.19	1.09
A2727	*Pseudomonas geniculata AT 19374* (99.78%)		39.7	1.23	0.79
A2731	*Enterobacter absuriae JM 6051* (99.49%)		44.3	1.18	1.15
A2733	*Bacillus subtilis subsp. inoquosorum KT 13429* (99.93%)		52.0	3.61	0.91
A2735	*Bacillus anthrasis ATT 14578* (99.85%)		44.3	4.30	2.04

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Supplementary Table 2. Degenerate primers used for end point PCR.

Gene	Primer Sequence (5'-3')	Name	Source
16S	AGAGTTTGATCCTGGCTCAG	Uni-27F	Weisburg et al.,1991
16S	GGTTACCTTGTTACGACTT	Uni-1492R	Weisburg et al.,1991
16S	GTGCCAGCMGCCGCGGTAA	U515F	Baker, et al.,2003
arsB	GGTGTGGAACATCGTCTGGAAYGCNAC	darsB1F	Achour et al.,2007
arsB	CAGGCCGTACACCACCAGRTACATNCC	darsB1R	Achour et al.,2007
ACR3(1)	GCCATCGGCCTGATCGTNATGATGTAYCC	dacr1F	Achour et al.,2007
ACR3(1)	CGGCG ATGGCCAGCTCYAAYTTYTT	dacr1R	Achour et al.,2007
ACR3(2)	TGA TCTGGGTCATGATCTTCCCVATGMTGVT	dacr5F	Achour et al.,2007
ACR3(2)	CGGCCACG GCCAGYTCRAARAARTT	dacr4R	Achour et al.,2007
arsC	TCGCGTAATACGCTGGAGAT	amlt-42-f	Sun et al.,2004
arsC	ACTTTCTCGCCGTCTTCCTT	amlt-376-r	Sun et al.,2004
arsC	TCACGCAATACCCTTGAAATGATC	smrc-42-f	Sun et al.,2004
arsC	ACCTTTTCACCGTCCTCTTTCGT	smrc-376-r	Sun et al.,2004
arsC	AGCCAAATGGCAGAAGC	P52F	Cavalca, et al.,2010
arsC	GCTGGRTCRTCAAATCCCCA	P323R	Cavalca, et al.,2010
arrA	CGAAGTTCGTCCCGATHACNTGG	AS1F	Song et al.,2009
arrA	GGGGTGCGGTCYTTNARYTC	AS1R	Song et al.,2009
arrA	GTCCCNATBASNTGGGANRARGCNMT	AS2F	Song et al.,2009
arrA	ATANGCCCARTGNCCYTGNG	AS2R	Song et al.,2009
aoxB	CCACTTCTGCATCGTGGGNTGYGGNTA	aoxBM1-2F	Quemeneur et al.,2008
aoxB	TGTCGTTGCCCCAGATGADNCCYTTYTC	aoxBM3-2R	Quemeneur et al.,2008
arsM	TCYCTCGGCTGCGGCAAYCCVAC	arsMF1	Jia et al.,2013
arsM	GTGCTCGAYCTSGGCWCCGGC	arsMF2	Jia et al.,2013
arsM	GGCATCGACGTGCTKCTBTCSGC	arsMF3	Jia et al.,2013
arsM	AGGTTGATGACRCAGTTWGAGAT	arsMR1	Jia et al.,2013
arsM	CGWCCGCCWGGCTTWAGYACCCG	arsMR2	Jia et al.,2013
arsM	GCGCCGGCRAWGCAGCCWACCCA	arsMR3	Jia et al.,2013

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Supplementary Table 3. Sample site description.

Air Temperature (°C)	Soil Temperature (°C)	Organic Matter 500°C (% active)	NO_3^- (ppm)	NH_4^- (ppm)	pH	S (ppm)	K (ppm)	Ca (ppm)	Mg (ppm)	Fe (ppm)	As (ppm)
13.3	57.4	7.1	4.6	1.7	8.0	28	37	2545	114	67.1	2.58

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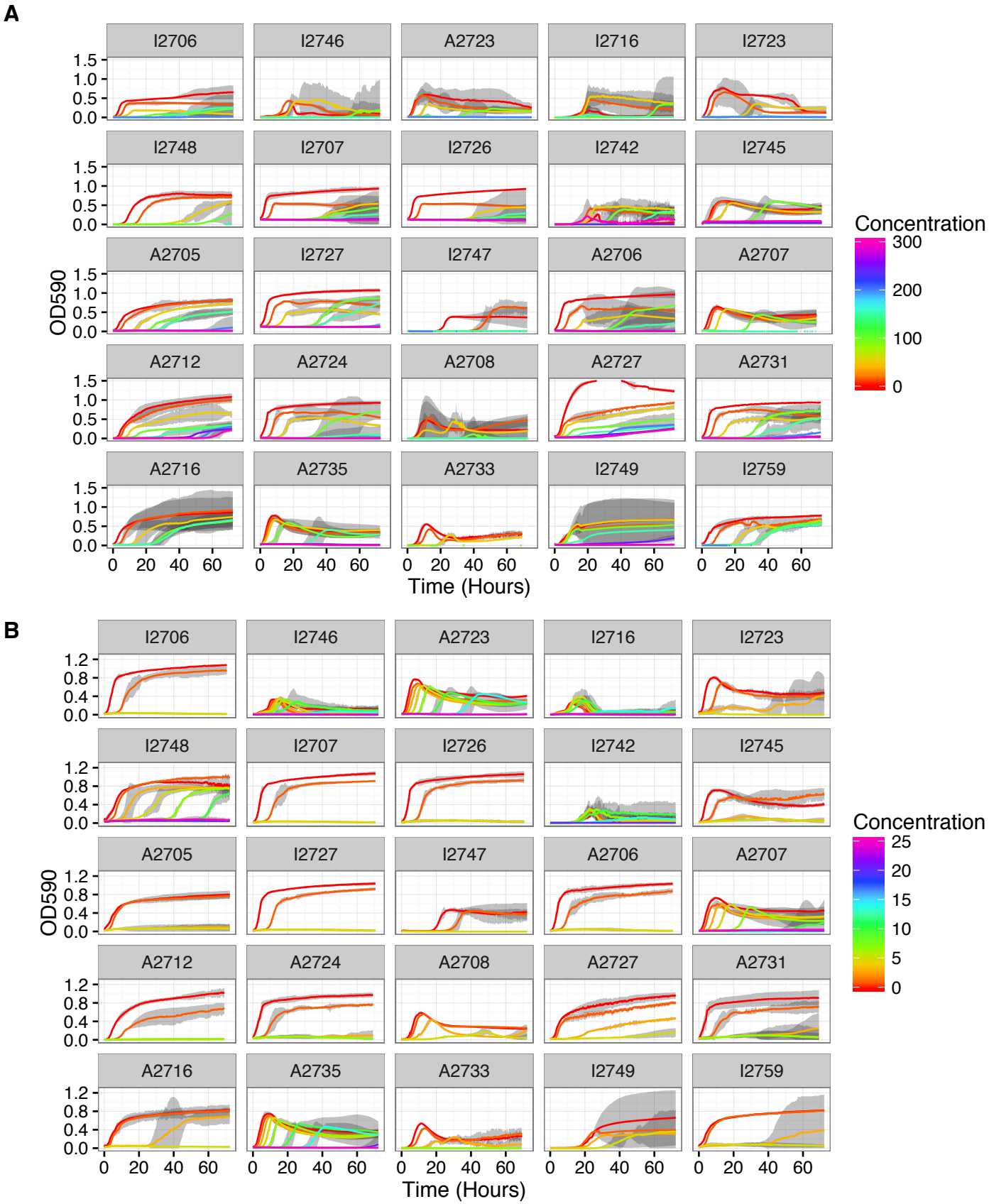
Supplementary Figure 1. Average OD590 over 72h in TSB50 with in increasing concentrations of arsenate (**A**) or arsenite (**B**). Grey ribbon represents 95% confidence intervals from three replicates. Note the difference in color scales.



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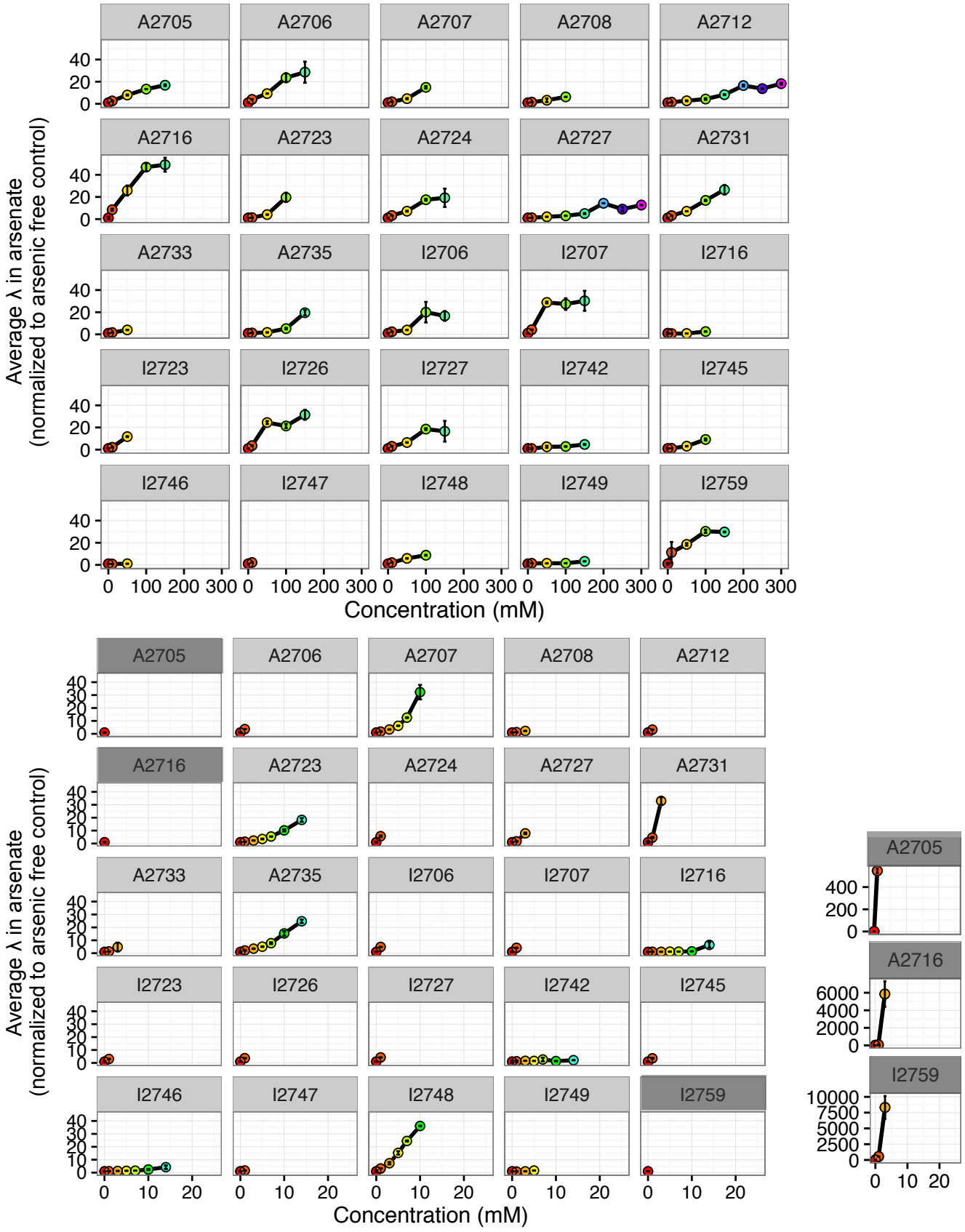
Supplementary Figure 2. Growth phenotypes in TSB50 with increasing concentrations of arsenate and arsenite normalized to growth in TSB50 without arsenic. Points are averages from three technical replicates, and error bars show standard deviation. Note the different scale for λ in arsenite for isolatesA2705, A2716, and I2759.



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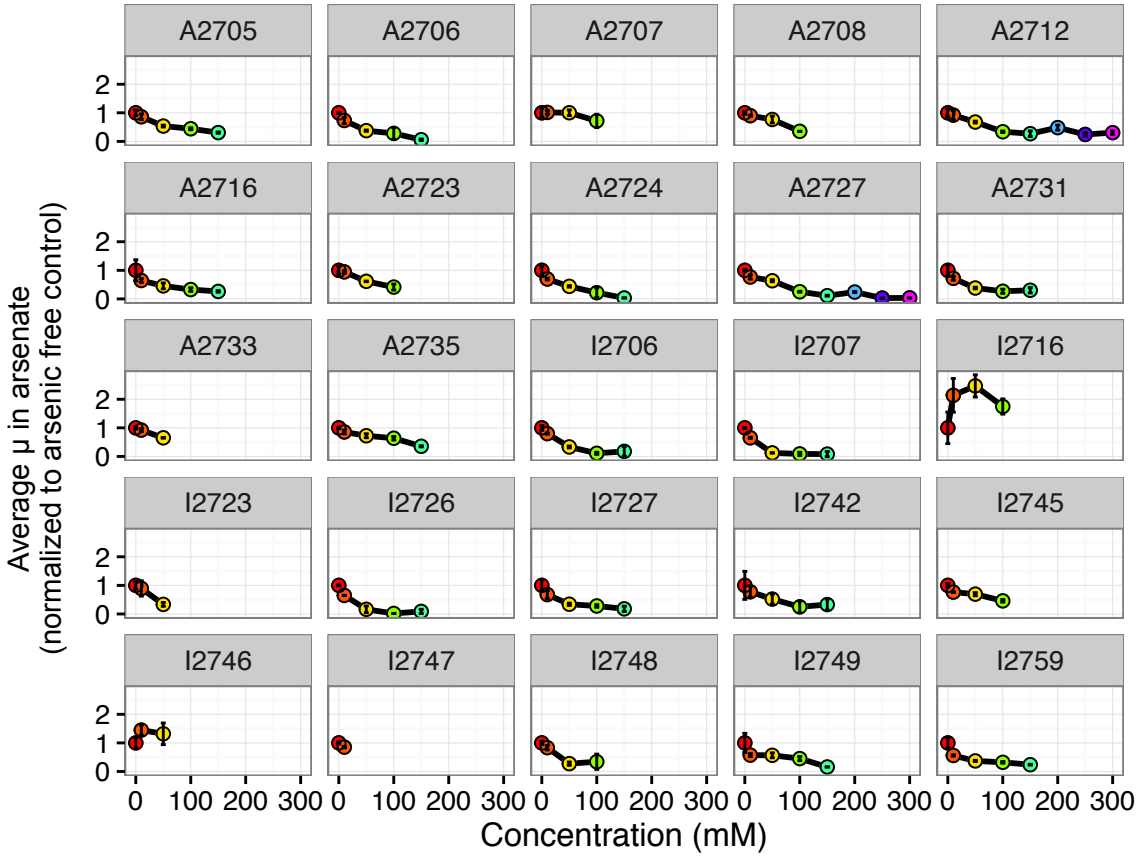

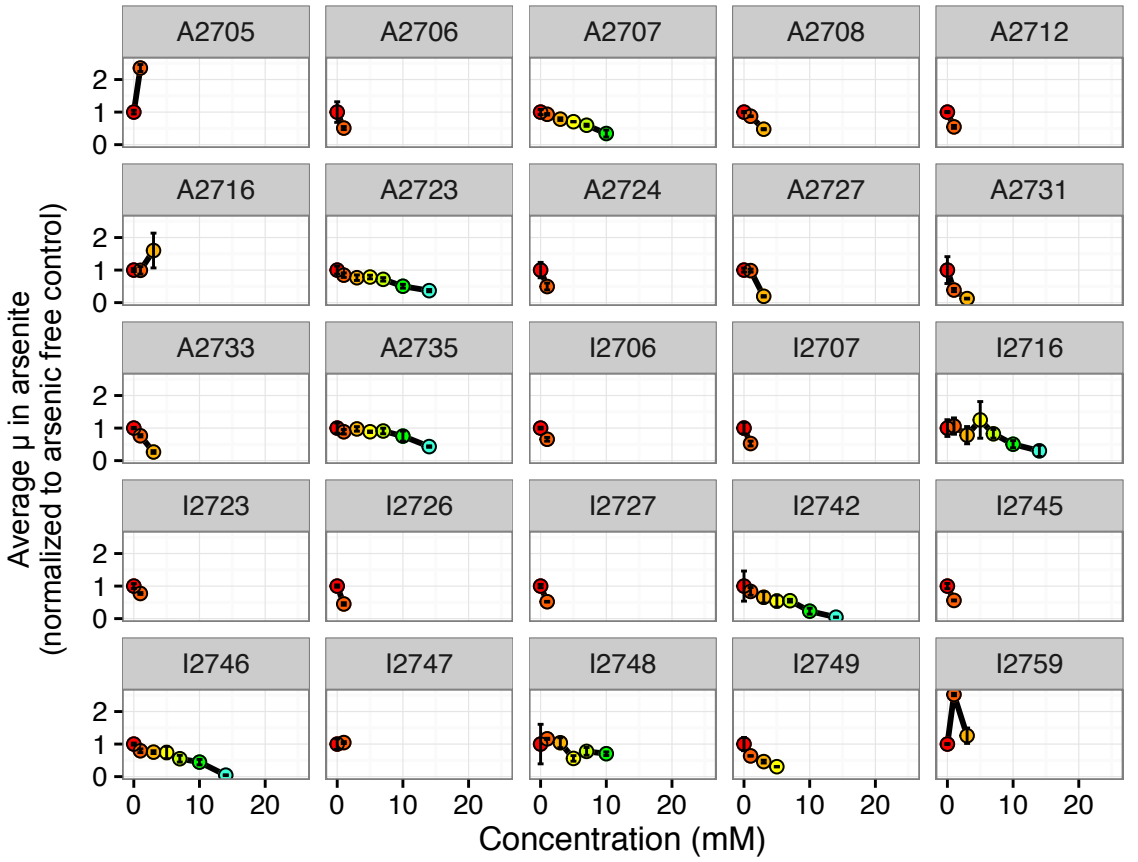

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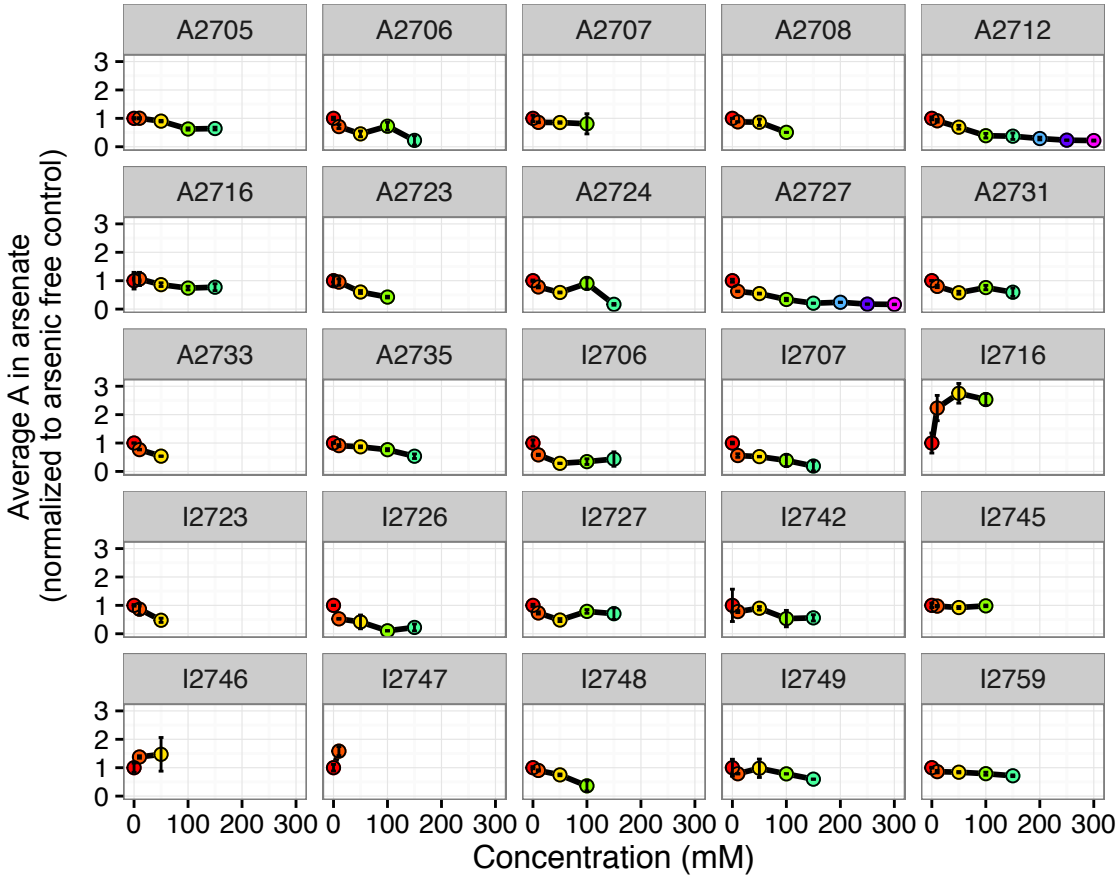

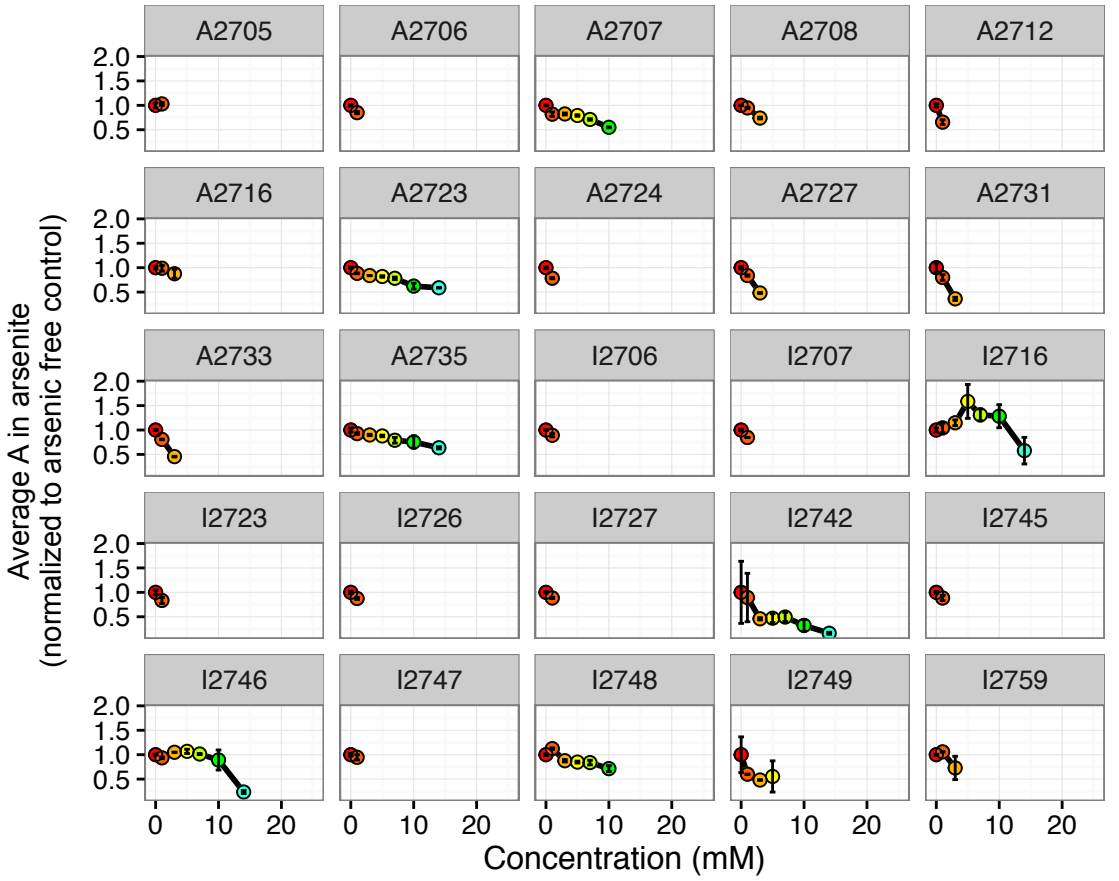

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