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Effects of Arsenic on Trichloroethene–Dechlorination Activities of *Dehalococcoides mccartyi* 195

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Abstract: Arsenic and trichloroethene (TCE) are among the most prevalent groundwater contaminants in the United States. Co-contamination of these two compounds has been detected at 63% of current TCE-contaminated National Priorities List sites. When in situ TCE reductive dechlorination is stimulated by the addition of fermentable substrates to generate a reducing environment, the presence of arsenic can be problematic because of the potential for increased mobilization and toxicity caused by the reduction of arsenate [As(V)] to arsenite [As(III)]. This study assesses the effects of arsenic exposure on the TCE-dechlorinating activities of *Dehalococcoides mccartyi* strain 195. Our results indicate that 9.1 μM As(III) caused a 50% decrease in *D. mccartyi* cell growth. While As(V) concentrations up to 200 μM did not initially impact TCE dechlorination, inhibition was observed in cultures amended with 200 μM As(V) and 100 μM As(V) in 12 and 17 days, respectively, corresponding with the accumulation of As(III). Transcriptomic and metabolomic analyses were performed to evaluate cellular responses to both As(V) and As(III) stress. Amendment of amino acids enhanced arsenic tolerance of *D. mccartyi*. Results from this study improve our understanding of potential inhibitions of *D. mccartyi* metabolism caused by arsenic and can inform the design of bioremediation strategies at co-contaminated sites.

Introduction

Trichloroethene (TCE) is a chlorinated organic that has been widely used as an industrial solvent for decades. Because of improper disposal, storage, and handling practices, TCE and its daughter-products of environmental transformation, that is, dichloroethene (DCE) and vinyl chloride (VC) are now ubiquitous groundwater contaminants of environmental and public health concern.(1) While numerous microorganisms have demonstrated the ability to reduce TCE, only *Dehalococcoides mccartyi* is known to convert TCE to the benign product ethene via reductive dehalogenation.
(2−7) Bioremediation of TCE by reductive dehalogenation using *D. mccartyi* strains has been well characterized, but there are substantial

knowledge gaps regarding the dechlorination of TCE under environmentally relevant conditions.

It is rare that chlorinated solvents are the only contaminants of concern at a contaminated site. For example, arsenic, one of the most frequent co-contaminants, has been reported at approximately 63% of current TCE-contaminated National Priorities List sites.(8) Arsenic originates from both anthropogenic and geogenic sources(9−12) and is present as a background constituent in many drinking water sources across the United States, even when not identified as a contaminant. (13) Arsenic is of particular interest as a co-contaminant in TCE-contaminated sites because of the critical effects of the oxidation state on arsenic toxicity and bioavailability.(14,15)

The toxicity mechanism for arsenate [As(V)] is the inhibition of phosphate-related functions because of the physical and chemical similarities between the two oxyanions.(16) The physical similarity to phosphate causes As(V) to enter cells through phosphate transporters. Detoxification of As(V) in bacteria is achieved through reduction of As(V) to As(III) and subsequent export from the cell,(17) and *D. mccartyi* strains have been reported to contain the arsenic toxicity genes required for this process.(18,19) Arsenite [As(III)] has been shown to cause toxicity to cells by binding to protein sulfhydryl groups, thereby inhibiting enzymatic catalysis.(20,21) Previous studies also reported that, while As(III) has a higher affinity for sulfhydryl groups, As(V) also interacts with them, but at much higher concentrations.(22)

There is a consensus in the literature that the solubilization of arsenic is most common during perturbed redox conditions (i.e., oxidative to reductive) which is typical during *in situ* reductive dehalogenation when fermentable substrates are amended to generate a reducing environment.(10,14,23) The effect of increasing aqueous arsenic concentrations on the organisms responsible for *in situ* reductive dehalogenation is unknown. However, past research has demonstrated that exposing TCE-reducing isolates and communities to various stressors (e.g., pH changes and sulfide accumulation) often results in the accumulation of the more toxic intermediates, DCE and VC. (24−26)

In this study, we investigated the effects of arsenic contamination on the dechlorination activity and gene expression in an axenic *D. mccartyi* strain 195 culture (Dhc195) in laboratory microcosms. To achieve this objective, we integrated results from physiological analyses, cell growth quantification, transcriptomic studies, and

extracellular metabolomic analyses after perturbation by As(III) or As(V).

Methods and Materials

Reagents

TCE, DCE, VC, and ethene (99.6%, ACS reagent) were obtained from Acros Organics (Geel, Belgium) or Sigma-Aldrich (USA). Sodium arsenate and sodium arsenite solutions were prepared from solid sodium arsenate dibasic heptahydrate and sodium (meta)arsenite, respectively, obtained from Sigma-Aldrich (USA). All other chemicals used were of reagent grade quality or higher.

Culture and Growth Conditions

Dhc195 was cultivated in defined mineral salt medium with an H_2–CO_2 (80:20 vol/vol) headspace as previously described.[7,27] Dhc195 was grown with 5 mM acetate as a carbon source, TCE as the electron acceptor in amendments of 77 µmol (with the number of amendments varying per experiment), H_2 provided in the headspace as the electron donor, and 0.5 mL of vitamin solution[28] containing 20 mg/L of vitamin B12 (final concentration 100 µg/L) as additional nutrients. All cultures were incubated without light or agitation at 34 °C for the duration of the experiment.

To study the effect of arsenic on the TCE-dechlorination activity, Dhc195 was grown with concentrations of As(V) or As(III) varying from 0 to 200 µM amended at the time of inoculation. Subsequent amendments of TCE were added to the cultures once TCE and cis-DCE were depleted. Experimental conditions were compared against positive biotic controls containing no arsenic. Abiotic controls with and without arsenic amendment showed neither TCE dechlorination or arsenic reduction (data not shown). All experiments were performed in triplicate.

Minimum Inhibitory Concentration Assay

To calculate the minimum inhibitory concentration (MIC) of As(III), Dhc195 was grown with concentrations of As(III) ranging from 5 to 30 µM As(III). As(III) was amended 24 h after inoculation. Cell samples were harvested every 24 h for 5 days. The MIC was defined as the concentration that caused a 50% decrease in the exponential growth rate as compared to the control condition and calculated as previously described.[29] To calculate the MIC for Dhc195 + folate and Dhc195 + amino acids, the above procedure was followed including the

amendment of folate (400 μg/L) or a 20-amino acid solution (20 mg/L each) at the time of inoculation, respectively.

DNA Extraction and Cell Enumeration

A 1.5 mL aliquot was sampled from each replicate for DNA extraction, and cells were pelleted by centrifugation ($21,000g$ 10 min at 4 °C). DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, USA) according to the manufacturer instructions for Gram-positive bacteria. Cell enumeration was performed using qPCR with SYBR-green-based detection agents. The qPCR primers used were strain-specific, targeting the reductive dehalogenase *tceA* gene as previously described.(30)

Differential Gene Expression Assay

To elucidate gene expression changes resulting from arsenic perturbation, two arsenic-amended conditions [10 μM As(III) and 100 μM As(V)] and a positive control containing no arsenic were analyzed. Arsenic was amended during the exponential growth of the cells when approximately 40% of the second dose of TCE had been consumed. Cells were collected approximately 5 h after amendment of arsenic. Each filter containing cells from one serum bottle was placed in a 2 mL microcentrifuge tube and frozen with liquid nitrogen and stored at −80 °C until processing. Cells from 24 serum bottles for each analyzed condition were collected via vacuum filtration (filter pore size 0.2 μm). RNA collection and purification were performed as previously described.(31)

Microarray data processing and analysis using the Affymetrix (USA) GeneChip microarray for measuring differential gene expression was applied as previously described.(32) The chip contains 4744 probe sets representing more than 98% of open reading frames from four published *D. mccartyi* genomes (strains 195, BAV1, VS, and CBDB1). cDNA was synthesized from 10 μg of RNA. Each cDNA sample was fragmented, labeled, and hybridized to an array. The procedures were performed according to the protocols established in Section 3 of the Affymetrix GeneChip Expression Analysis Technical Manual (http://www.affymetrix.com). Microarray data analysis methods were performed using the "affy" Bioconductor package as previously described.(33−36) Probe set hybridization signal intensities were calculated using the "mas5" function from the "affy" package. Statistical significance of individual differentially expressed transcripts is defined as fold-change > 2, *p*-value < 0.05, and average signal intensity of either control or experimental condition >200. Significance

of differential expression of metabolic pathways was determined using the Wilcoxon rank-sum test as previously described.(37)

Extracellular Metabolomics Analysis

For extracellular metabolomic analysis, the same three conditions were studied as in the differential gene expression assay [10 µM As(III), 100 µM As(V), and positive control]. Samples were taken at two time points during the experiment (when approximately 45 and 95% of the second TCE dose had been consumed, corresponding to 25 and 48 h after amendment of the second TCE dose), at which point 1 mL of liquid medium was withdrawn from the culture and cells were removed via centrifugation (21,000g 10 min at room temperature ≈ 23 °C) and stored at −80 °C until analysis. The first time point (approximately 45% TCE consumed) corresponds to the transcriptomic sample time point which was taken 5 h after 40% of TCE was consumed.

Targeted and untargeted metabolomic data were acquired using gas chromatography time-of-flight mass spectrometry (GC-TOF-MS), processed, and normalized at the West Coast Metabolomics Center (University of California, Davis, CA, USA) as previously described. (38) Statistical significance for this analysis was defined as changes exhibiting fold-change > 2, p-value < 0.05, and average signal intensity of either control or experimental condition >500.

Untargeted Metabolite Identification

In silico determination of candidate metabolites was performed with the workflow in Figure S1. Briefly, we subtracted one and two trimethylsilyl (TMS) moieties from the mass spectrum and found matching m/z ions from the one and two TMS subtraction. The matched m/z ions and abundances were compared against two different mass spectrum databases—MassBank of North America (MoNA; http://mona.fiehnlab.ucdavis.edu/) and CFM-ID (http://cfmid.wishartlab.com)(39)—to search for potential candidate metabolites. The workflow was validated with target metabolites (e.g., threonine) treating them as unknowns.

Calibration standards for candidate metabolites were prepared from the high-performance liquid chromatography (HPLC) grade crystalline solid. Candidate metabolites were quantified using liquid chromatography/mass spectrometry (LC–MS/MS) using a 1260 Infinity II LC (Agilent Technologies, USA) and 6460 Triple Quad MS (Agilent Technologies, USA) equipped with an HILIC Plus 3.5 µm (4.6 mm × 100 mm) column (Agilent Technologies, USA). The mobile phase was 50% 10 mM ammonium acetate in LCMS grade water and 50% 10 mM

ammonium acetate in LCMS grade methanol and was run isocratically. The analyses were conducted at a flow rate of 0.4 mL/min. The injection volume was 25 μL. Time of data acquisition was 4 min per sample. The MS source parameters were as follows: gas temperature (325 °C), sheath gas temperature (350 °C), gas flow (9 L min^{-1}), sheath gas flow (9 L min^{-1}), nebulizer (25 psi), nebulizer gas (nitrogen), and capillary voltage (ESI$^+$ mode 3.5 kV; ESI$^-$ mode 3 kV). Multiple reaction monitoring transitions for the candidate metabolites are shown in Table S1.

Analytical Methods

Chlorinated ethenes and ethene were measured by injecting 100 μL of the headspace gas from the cell cultures on an Agilent 7890A gas chromatograph equipped with a flame ionization detector and a 30-m J&W capillary column with an inside diameter of 0.32 mm (Agilent Technologies, USA). A gradient temperature program method was used as previously described.(40)

Measurement of soluble As(III) and As(V) was performed using an Agilent 1260 Infinity high-performance liquid chromatograph (Agilent Technologies, USA) equipped with an Aminex HPX-87H Ion Exclusion Column (300 mm × 7.8 mm, Bio-Rad, USA) coupled to a Hamilton PRP-X300 Reversed-Phase Column (250 mm × 4.1 mm, Hamilton Co., USA) and an Agilent 7700 series inductively coupled plasma mass spectrometer (Agilent Technologies, USA) as previously described. (41) Arsenous acid, arsenic acid, monomethylarsonate, dimethylarsinate, and arsenobetaine are quantifiable using the applied method.(41) Samples consisting of 0.5 mL of the liquid medium were collected at three time points during the experiment (day 0, day 12, and day 17) from both As(III)- and As(V)-amended cultures. Liquid medium samples were filtered using 0.2 μM HPLC-grade syringe filters (Pall Life Sciences, England) and diluted to obtain analytical concentrations within standard solution calibration.

Results and Discussion

Inhibition by Arsenic of TCE-Dechlorination Activity and Cell Growth of Dhc195

The effects of As(V) and As(III) on growth and TCE dechlorination of Dhc195 were evaluated in concentrations ranging from 5 to 200 μM and 5 to 30 μM, respectively. The presence of As(V) did not initially inhibit Dhc195 growth. Rates of TCE dechlorination in cultures amended with 100 and 200 μM As(V) were not significantly different

from the control containing no As(V) during consumption of the first two amendments of TCE (Figure 1A). After the third amendment of TCE on day 10, a decrease in the dechlorination rate of Dhc195 was observed in cultures amended with 200 μM As(V), and 38% of TCE (27.1 ± 6.0 μmol TCE/bottle) from the third amendment remained at the end of the experiment on day 17. After the fourth amendment of TCE on day 12 in cultures amended with 100 μM As(V), the dechlorination rate of Dhc195 decreased, and 36% of TCE (27.1 ± 12.1 μmol TCE/bottle) remained at the end of the experiment (day 17). There were no significant differences in final cell number of Dhc195 in cultures containing 100 or 200 μM As(V) as compared to the control (Figure S2). The similarities in cell number are not surprising, as it has been previously reported that most of the growth in Dhc195 microcosms occurs during consumption of the first TCE amendment— the exponential growth rate of Dhc195 for the first amendment of TCE was 0.21/day and decreased to 0.05/day during amendments 2 through 4.(34) This indicates that most cell growth would have occurred during the consumption of the first dose of TCE before As(III) accumulated and disrupted cellular function.
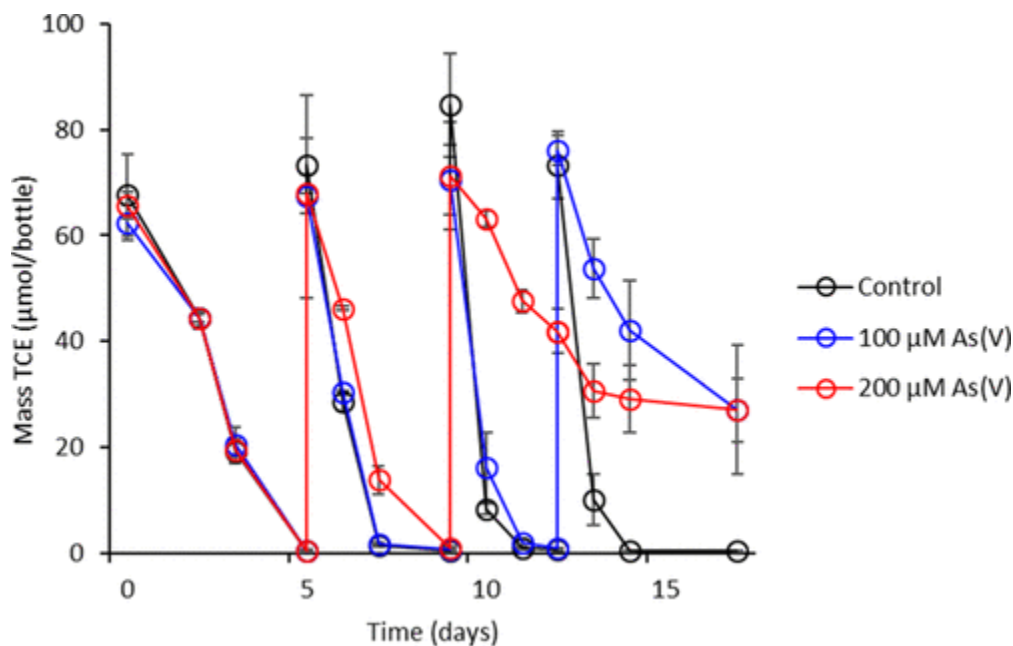
Figure 1

Figure 1. TCE consumption by Dhc195 amended with 0, 100, and 200 μM As(V). Additional TCE was amended on days 5, 10, and 13. Error bars represent one standard deviation of experimental triplicates.

Modification of the arsenic species by Dhc195 was monitored during inhibition experiments. In cultures amended with 100 μM As(V), 23% of

As(V) was reduced to As(III) by day 17, with all observed reduction of As(V) occurring between day 12 and day 17 (Figure 2A). In cultures amended with 200 μM As(V), 19% of total As was reduced to As(III) by day 12 and 54% of total As was reduced by day 17 (Figure 2B). These observed As(V) reduction rates were significantly greater than those observed in abiotic controls (Figure 2C) as well as the published abiotic As(V) reduction rates under similar conditions,(42) indicating the generation of As(III) was due to the detoxification functions of Dhc195.
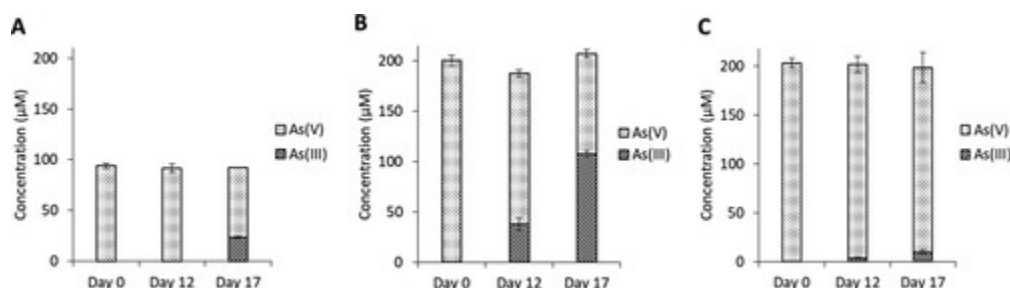
Figure 2

Figure 2. Concentrations of aqueous As(III) and As(V) on days 0, 12, and 17 in Dhc195 cultures amended with (A) 100 μM As(V), (B) 200 μM As(V), and (C) abiotic control amended with 200 μM As(V). Error bars represent one standard deviation of experimental triplicates.

The genomes of *D. mccartyi* strains contain a suite of arsenic resistance genes, including *arsR*, *arsB*, *arsC*, and *arsM* (DET1005, 0908, 0143, 1420), encoding the functions of reducing As(V) to As(III), arsenic methylation, and transporting As(III) out of the cell as pathways of arsenic detoxification.(18) Arsenic "detoxification" was observed here via the reduction of As(V) to As(III) by Dhc195, as Dhc195 does not carry the genes required for dissimilatory As(V) reduction, arrA, and arrB.(18,43) Even though the amended As(V) concentrations did not initially affect metabolic function of Dhc195, the so-called detoxification mechanism caused the accumulation of the more toxic As(III), which inhibited TCE dechlorination.

Physiological experiments were performed to quantify the impacts of As(III) on Dhc195. Because of more potent toxic properties of As(III) as compared to As(V) in a variety of organisms,(16,44,45) concentrations of As(III) ranging from 5 to 30 μM were applied. In the positive control samples, complete reduction of 87.1 ± 2.3 μmol TCE occurred within 5 days (Figure 3A). In comparison, cultures containing 5, 7.5, 10, 20, and 30 μM As(III) only reduced 78% (63.4 ± 8.7 μmol), 65% (56.2 ± 2.9 μmol), 42% (33.9 ± 7.8 μmol), 26% (20.7 ± 4.6 μmol), and 21% (18.0 ± 9.3 μmol), of TCE, respectively. To determine the MIC of As(III) for

Dhc195, cell numbers were quantified every 24 h over a 5-day experiment under various As(III) concentrations. The MIC for Dhc195 was calculated to be 9.1 µM As(III) (Figure 3B).

Figure 3

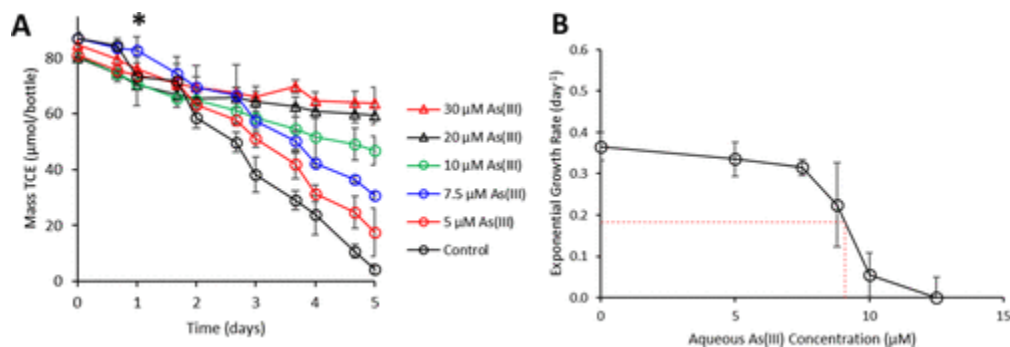Figure 3. (A) TCE consumption (µmol/bottle) by Dhc195 amended with a range of As(III) concentrations (0–30 µM). The asterisk denotes the time at which As(III) was amended to the cultures. (B) Exponential growth rates (per day) of Dhc195 amended with increasing As(III). The red dashed line indicates the MIC of As(III) for Dhc195. Error bars represent one standard deviation of experimental triplicates. The absence of error bars indicates bars are smaller than the marker.

In experiments when 200 µM As(V) was amended [rather than As(III)], Dhc195 was able to continue to dechlorinate TCE even when generated aqueous As(III) concentrations reached 38 µM, suggesting that Dhc195 was able to develop a higher As(III) tolerance under gradual accumulation as compared to the pulse input. In cultures amended directly with As(III), no change in the oxidation state of arsenic was observed (Figure S3).

Differential Gene Expression Resulting from Arsenic Inhibition in Dhc195

Transcriptomic analysis was performed to identify genes with significant changes in expression in both As(III)- and As(V)-amended cultures as compared to the positive controls. In 10 µM As(III)-amended cultures, 52 transcripts were down-regulated and 278 transcripts were upregulated. In cultures amended with 100 µM As(V), 33 transcripts were down-regulated and 332 transcripts were significantly upregulated. The differentially expressed genes were categorized by module based on their annotation in the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Figure 4). Genes not categorized in the KEGG system or identified as hypothetical proteins were not included in Figure 4. Not surprisingly, exposure to As(III) resulted in more down

regulated genes (Figure 4A) compared to As(V) exposure, which mostly resulted in the upregulation of genes (Figure 4B). There is more overlap in upregulated genes than in downregulated genes in the different treatments (Figure 4C). A table of significantly up and downregulated genes after amendment of 10 µM As(III) and 100 µM As(V) relevant to the discussion can be found in Tables S2 and S3, respectively.
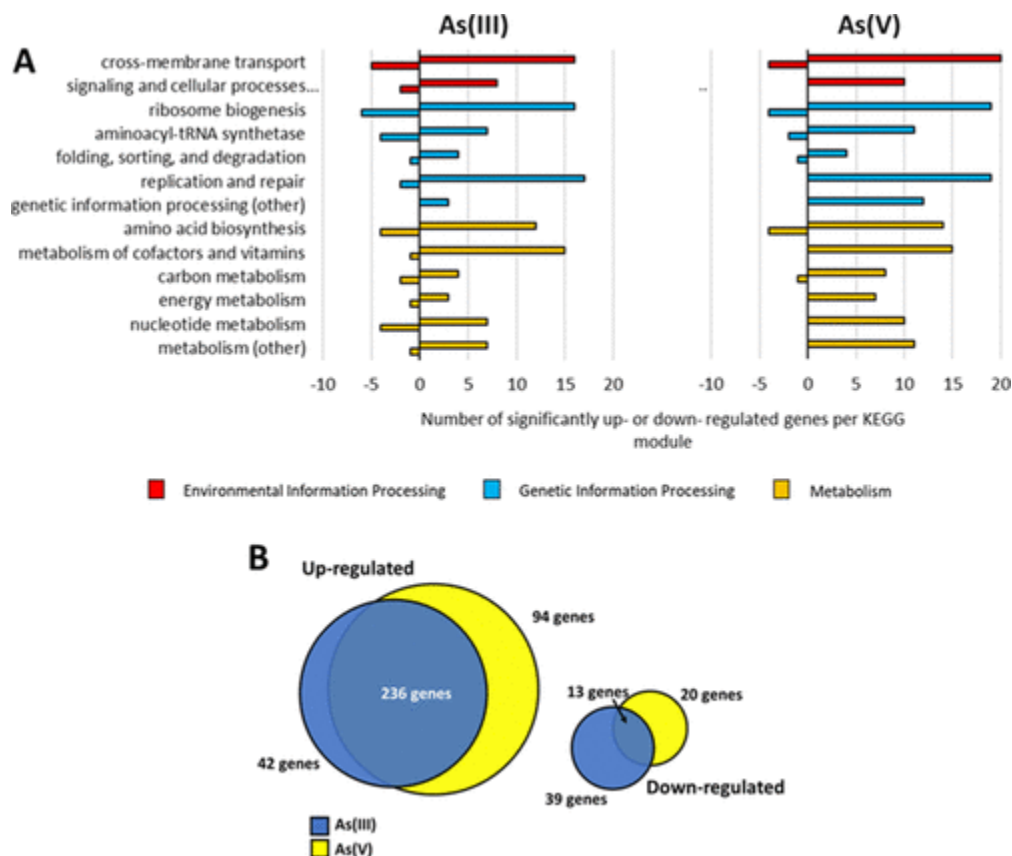
Figure 4

Figure 4. Number of significantly up and downregulated genes in Dhc195 organized by the KEGG module in cultures amended with (A) 10 µM As(III) and 100 µM As(V). Genes that were identified to code for hypothetical proteins and genes not identified in the KEGG database are not shown. Positive numbers denote genes upregulated while negative numbers denote genes downregulated. The colors of the bars indicate the KEGG structural complex category of each module. (B) Graphical representation of quantification of up and downregulated genes that were found to be shared and distinct between the As(III)- and As(V)-amended conditions.

The reductive dehalogenase gene responsible for dechlorination of TCE to VC in Dhc195, *tceA* (DET 0078–0079), did not exhibit a significant

differential expression in response to As(III) or As(V). The *arsR* family transcriptional regulation gene (DET 1005) exhibited significant upregulation in both As(III)- and As(V)-containing cultures. However, neither the cytoplasmic arsenate reductase *arsC* (DET0143) or the arsenite efflux transporter genes (DET0908) exhibited a differential expression at the time of the sample collection, suggesting it may take more time after exposure to arsenic for these genes to exhibit differential expression. It is also possible that this transcriptional regulator is induced by the presence of arsenic but has a different role in genetic regulation for Dhc195 *arsC* repression, as *arsR* has been previously characterized as a regulator which
represses *arsC* expression unless arsenic is present to inhibit the repressor and induce *arsC* expression.(46) It is important to note that the *arsC* enzyme is usually expressed at low levels because of the leaky *ars* operon promoter,(47) and is expressed in the positive control, As(III)-amended, and As(V)-amended conditions even though no differential expression was observed. The arsenite methyltransferase *arsM* (DET1420) was upregulated in both As(III)- and As(V)-amended cultures, but no production of methylated arsenic compounds were observed. It is possible that this gene is misannotated in the Dhc195 genome and the substrate for this methyltransferase is not arsenic.

Both As(III)- and As(V)-containing compounds have been shown to promote DNA mutagenesis in bacteria.(48) Because of this, and general stress response, it is unsurprising that genes involved in DNA repair and replication, translation, and nucleotide metabolism were found to be upregulated in this study (Figure 4). Genes involved in membrane transport may be upregulated to serve multiple purposes, including export of potential stress-signal molecules and import of necessary biomolecules for assimilation from the environment. Genes involved in folate biosynthesis were observed to be upregulated under As(III)- (DET0016, 1603–1604) and As(V)- (DET0016, 1603, 1605) amended conditions. Biosynthesis of arginine (det00220 per KEGG pathway database) was observed to be upregulated in both As(III)- and As(V)-containing cultures (p-value = 0.035 and p-value = 0.044, respectively), and the valine, leucine, and isoleucine biosynthesis pathway (det00290) was observed to be upregulated in As(III)-containing cultures (p-value = 0.047). The prolyl-tRNA, isoleucyl-tRNA, and tryptophanyl-tRNA synthetases (DET0368, 1038, 1343) were also found to be upregulated in both As(III)- and As(V)-containing cultures, while histidyl-tRNA and tyrosyl-tRNA synthetases were found to be downregulated under both conditions.

Significant changes in the abundance of aminoacylated-tRNA molecules were also identified by microarray analysis. In cultures amended with As(V), glutamyl-tRNA and prolyl-tRNA were found in significantly higher abundance as compared to the control. In culture amended with As(III), glycyl-tRNA and threonyl-tRNA were found in significantly higher abundance as compared to the control. In both As(III)- and As(V)-amended cultures, arginyl-tRNA, alanyl-tRNA, histidyl-tRNA, methionyl-tRNA, and seryl-tRNA were found in significantly lower abundance as compared to the control. In As(V) containing cultures, asparaginyl-tRNA was also found in lower concentrations. Virtually all aminoacyl-tRNA synthetase enzymes that have been biochemically characterized contain sulfhydryl groups, and previous studies have identified tRNA synthetases as enzymes that can be affected by sulfhydryl group inhibitors.(49−51) The results of this study, showing higher abundances for some aminoacyl-tRNA molecules in the presence of arsenic and lower concentrations for others, suggest that arsenic has greater impacts on some aminoacyl-tRNA synthetases than others in Dhc195. Numerous aminoacyl-tRNA synthetases in other bacteria have been identified to be resistant to inhibition by sulfhydryl-inhibiting agents, including the lysyl-tRNA synthetase from *Escherichia coli* and the methionyl-tRNA synthetase from *Sarcina lutea*. (52,53) Biochemical characterization of tRNA synthetases in Dhc195 is required to draw further conclusions regarding the sensitivity of certain synthetases to sulfhydryl group inhibitors.

Of the differentially expressed genes observed herein, 236 upregulated genes and 13 downregulated genes were shared between As(III)- and As(V)-amended cultures (Figure 4C), suggesting certain cellular responses in the presence of 10 μM As(III) and 100 μM As(V) in Dhc195 are shared while others are specific to As(III) or As(V). It is possible that the observed similarities in genetic response to As(III) and As(V) are a result of small amounts of cytoplasmic reduction of As(V) to As(III) by Dhc195 (lower than the limit of detection) and the subsequent presence of As(III) since *arsC* is always slightly expressed. Additionally, both As(III) and As(V) have been shown to induce heat-shock response proteins such as *dnaJ*, *dnaK*, and *grpE* in bacteria *Leuconostoc esenteroides*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*. (54−56) However, these stress-response genes were not upregulated in Dhc195 under As(III) or As(V) stress in this study (DET1399-1400, 1411). Arsenic is also known to interact with sulfur-containing enzymes directly, which may impact multiple cellular processes.(20−22) Iron–sulfur clusters are common in bacterial proteins, and a variety of proteins in Dhc195 contain iron–sulfur units, including reductive

dehalogenases(4) and formate dehydrogenase (DET0112),(57) the latter of which was upregulated under As(III)-amended conditions. Because As(III) and As(V) are otherwise known to act via different modes of toxicity, many of these shared gene expression changes may be related to the general stress response in Dhc195.

In As(V)-amended cultures, many of the upregulated genes distinct from As(III)-amended cultures coded for proteins known to interact with phosphate groups, including kinases (DET0049, 0405, 0744) and phosphatases (DET0713, 0797). As(V) has been previously shown to act as a competitive inhibitor with phosphatases,(17) and the upregulation of kinases may be a transcriptional response to the tendency of arsenate to form ADP-arsenate complexes, inhibiting formation of ATP.(57−59) Other genes upregulated solely in As(V)-amended cultures included genes for potassium uptake (DET0026-0027, 0029).

The increase in tolerance to As(III) after gradual accumulation as opposed to a pulse input observed in the physiological experiments in this study may be explained by the high number of differentially expressed genes shared between As(III)- and As(V)-amended cultures. It is possible that by the time As(III) began to accumulate to toxic levels in the cell, many of the required stress-response genes had been upregulated and the resulting cellular changes had already taken effect. This response may illustrate a more environmentally relevant system more closely than a pulse input of As(III). However, the kinetics of As(V) reduction in an environmental system could potentially be much faster than that observed herein, as As(V)-respiring bacteria are common in subsurface environments.(60)

Extracellular Metabolite Profile of *D. mccartyi* in the Presence of Arsenic

Both targeted and untargeted metabolomic analyses were applied to culture supernatant samples taken at 2 time points in cultures amended with no arsenic (positive control), 10 μM As(III) and 100 μM As(V). Time point 1 represents samples taken at the same time as the transcriptomic analysis and time point 2 represents samples taken 24 h after transcriptomic analysis, when 95% of TCE had been consumed. Significant changes in metabolite abundance resulting from amendment of As(III) and As(V) were identified at both time points (Figure 5).
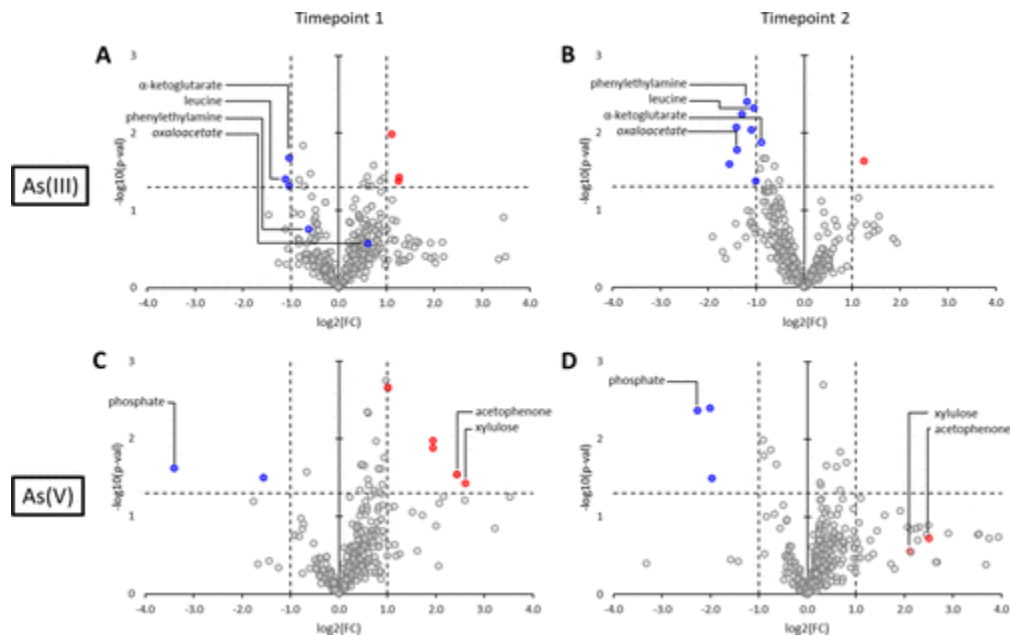
Figure 5

Figure 5. Volcano plots showing changes in metabolite abundance in the extracellular metabolite pool in Dhc195 amended with As(III) at time points (A) 1 and (B) 2 and Dhc195 amended with As(V) at time points (C) 1 and (D) 2 with log base 2 of the fold change as (arsenic/control) on the *x*-axis and negative log base 10 of the *p*-value on the *y*-axis. The horizontal and vertical dotted lines indicate the cutoffs for statistical significance. The colored markers indicate statistically significant metabolites that were present in higher (red) or lower (blue) abundances after arsenic amendment. Metabolites that were statistically significant under only one time point are colored in both time point figures for reference. Significant targeted metabolites are labeled and significant untargeted metabolites that were identified and experimentally validated are labeled in italics. Colored markers without labels denote untargeted metabolites that could not be identified.

In cultures amended with As(III) at time point 1 (Figure 5A), α-ketoglutarate and leucine were found to be present in lower abundance as compared to the control. Ketoglutarate is an intermediate in the central carbon assimilation pathway of Dhc195 and a precursor to glutamate.(61) At time point 2 (Figure 5B), 88% of all observed changes were decreases in abundance including all but one significant change. Leucine and phenylethylamine were observed to be significantly diminished in the supernatant. One untargeted metabolite significantly diminished in the supernatant under As(III)-stress was identified to be oxaloacetate, an intermediate in the tricarboxylic acid cycle and a precursor to glutamate, aspartate, and homocysteine in

Dhc195.(62) Tyrosine, isoleucine, fumaric acid, and malic acid were also observed to be decreased, while not statistically significant. The decrease in extracellular metabolites from time point 1 to 2 suggests that Dhc195 imported many metabolites from the environment in concordance with the upregulation of ABC transporters and an ion channel (DET0224, 0795, 0814, 0817, 0851, 0856, 1179–1180, 1491, 1579) observed at time point 1. The observed decrease could also be the result of the decreased export of certain metabolites by Dhc195, as five genes related to membrane transport were downregulated in As(III)-amended cultures (DET0034, 0138, 0784, 1125, 1493). The transcriptomic analysis at time point 1 identified the tyrosine, isoleucine, and leucine biosynthesis pathways as significantly upregulated, indicating Dhc195 utilized extracellular import as well as upregulated biosynthesis to obtain these amino acids in the presence of As(III). Additionally, malic acid and fumaric acid are both precursors to multiple amino acids in the biosynthesis pathway of Dhc195, (61) and phenylethylamine is an intermediate in the phenylalanine metabolism.

At time point 1 in cultures amended with As(V) (Figure 5C), extracellular phosphate was found to be in lower abundance, whereas xylulose, an intermediate in pentose interconversions,(63) and acetophenone, an intermediate in phenylalanine biotransformation, (64) were observed to increase in abundance. In As(V)-amended cultures at time point 2 (Figure 5D), phosphate and shikimic acid (a folate precursor) were observed to be diminished in the supernatant, however the change in shikimic acid was not found to be statistically significant. As(V) is known to interact with phosphate-related cellular processes, which would induce Dhc195 to increase import of phosphate in the presence of As(V). This increased importation results in higher uptake of As(V) as well because the transporter responsible for phosphate also transports As(V) with varying specificity, (65,66) perpetuating the cytoplasmic reduction of As(V) to As(III) in Dhc195 and leading to As(III) toxicity. As As(III) accumulates, Dhc195 exhibits the gene regulation and import of amino acids and carbon pathway intermediates observed herein to counteract As(III) toxicity.

Only ten of the common amino acids were detected using this method (alanine, cysteine, glycine, isoleucine, leucine, methionine, proline, serine, threonine, tyrosine, and valine). Arginine was not reported using this method, as it is broken down into ornithine during sample analysis. The remaining nine (asparagine, aspartate, glutamate, glutamine, histidine, lysine, phenylalanine, serine, and tryptophan)

were present in too low abundance for detection in the supernatant. It has been previously asserted that Dhc195 almost exclusively synthesizes lysine, aspartate, and glutamine when these amino acids are provided in excess in growth medium of laboratory culture. (67) These amino acids were not found to be present in the extracellular metabolite profile in this study, consistent with their uptake by Dhc195 and the selectivity of ABC-transporters in Dhc195, (67,68) supporting the hypothesis that Dhc195 does possess selective ABC-transporters for amino acids. Based on these results, amino acids, anabolism intermediates, and folate may be important to Dhc195 under arsenic stress. A comprehensive table of changes in targeted and identified untargeted metabolites after amendment of 10 µM As(III) and 100 µM As(V) can be found in Tables S4 and S5, respectively.

MIC Experiments with Amendment of Folate or Amino Acids

After transcriptomic and metabolomic analyses, folate and amino acids were identified as metabolites of interest that may increase the arsenic tolerance of Dhc195 in arsenic-amended cultures. As(III) MIC experiments in Dhc195 were subsequently performed with amendment of 400 µg/L of folate and with a 20-amino acid solution (20 mg/L each). Exponential growth rates of Dhc195 with increasing As(III) were compared with exponential growth rates of Dhc195 + folate and Dhc195 + amino acids (Figure 6). The estimated MIC of As(III) for Dhc195 + folate and Dhc195 + amino acids were 6.8 µM As(III) and 12.2 µM As(III), respectively.

Figure 6
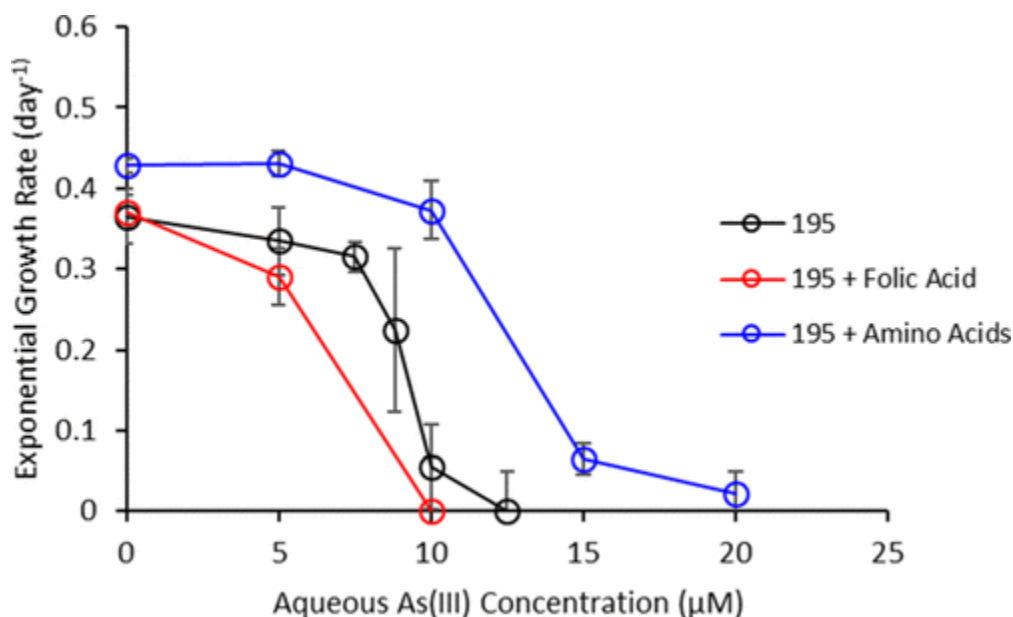
Figure 6. Exponential growth rate (day^{-1}) with increasing As(III) concentrations for Dhc195, Dhc195 amended with 400 µg/L of folic acid, and Dhc195 amended with amino acids (20 mg/L each of the 20 common amino acids). The MICs for Dhc195, Dhc195 + folate, and Dhc195 + amino acids were 9.1, 6.8, and 12.2 µM As(III), respectively. Error bars represent one standard deviation of experimental triplicates.

Results indicate that while amendment of folate did not enhance the tolerance of Dhc195 for As(III), the amendment of the 20-amino acid solution achieved this. Zhuang et al. demonstrated the uptake and utilization of exogenous amino acids by Dhc195 under regular growth conditions.(67) Dhc195 was observed to exhibit higher growth rates with the amendment of exogenous amino acids, which is verified in this study (Figure 6—time point: day 0). This metabolomic analysis along with the transcriptomic analysis indicates Dhc195 uptakes or retains amino acids and their biosynthesis intermediates to combat arsenic toxicity, likely due to inhibition of enzymatic catalysis by sulfhydryl-containing enzymes.

Folate is an important biomolecule, impacting DNA stability and serving as a precursor to DNA, RNA, and various amino acids including methionine.(69,70) Numerous studies have observed enhanced expression of folate biosynthesis genes, including *folE*, resulting from various cell stressors (e.g., temperature stress and antibiotic stress). (71) Waller et al. demonstrated a folate requirement, most likely tetrahydrofolate, in the catalytic activity of *ygfZ*, a tRNA-modifying enzyme involved in the assembly or repair of iron–sulfur clusters. (72) Waller et al. also observed a correlation between inactivation of folate biosynthesis genes and lowered activity of tRNA modification enzyme *miaB*.(72) The upregulation of both folate biosynthesis and *miaB*, an iron–sulfur cluster protein,(73) in Dhc195 in response to arsenic stress observed in this study suggests that Dhc195 may produce more folate to support repair of iron–sulfur clusters inhibited by arsenic interaction with sulfide-containing enzymes, including aminoacyl-tRNA synthetases. However, amendment of folate to Dhc195 cultures did not increase resistance to As(III) toxicity as measured by the growth rate. It is possible that Dhc195 does not have a folate transport system, as none is annotated in the genome. (18) Further studies are required to elucidate the importance of the folate biosynthesis pathway in Dhc195 under arsenic stress.

This study has demonstrated that the presence of As(III) or As(V) in a groundwater aquifer could lead to inhibition of TCE dechlorination by Dhc195. Based on these results, engineered bioremediation strategies,

such as retarding As(V) reduction rates and/or providing the TCE-dechlorinating community with exogenous amino acids could promote robust TCE dechlorination at arsenic contaminated sites. Future studies should build upon the complexity of the system (e.g., analysis of multi-member consortia) to develop a comprehensive understanding of the impacts of co-contaminants on TCE reductive dechlorination.

Acknowledgements:

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