Screening for Arsenate and Arsenite Resistant Microbes In Passive Treatment Systems for Metal Leachate Remediation

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Abstract

Bacteria play an important role in the biogeochemical cycle of metals in the environment, and their capabilities and mechanisms in transforming toxic metals are of significant interest in the environmental remediation of leachate. With this goal in mind, a passive treatment system at Trail (B.C) was built to remove metals (As, Zn, Cd) from landfill leachate. In this study, microbes were identified from the passive treatment system and their responses to arsenate and arsenite were investigated. Three approaches were applied; first, Enrichment cultures were established in order to identify microorganisms capable of reducing or oxidizing arsenic compounds. In this approach arsenic species and concentrations were monitored over the time. In the second approach, a metagenomic library was screened to identify functional genes putatively involved in various arsenic biotransformation processes. Thirdly, large DNA insert fosmid clones were screened for arsenate and arsenite resistance to allow sequencing gene encoding for arsenate biodetoxification .This high throughput and automated approach allows for rapid screening of libraries harbouring DNA with broad taxonomical diversity.

Key Words: arsenic resistance genes, microbial community, enrichment cultures, bioremediation, phylogeny, mining

1. Introduction

In the metallurgical industry and in mining, arsenic contamination is of great concern due to its toxicity and persistence in the ecosystem. Arsenic exists in four oxidation states: As(V), As(III), As(0) and As(-III). In aquatic environments, arsenic exists in the form of arsenate As(V) or arsenite As(III). The former species is the less toxic and less soluble form of arsenic oxyanion. In an aerobic environment, arsenate is dominant and can precipitate when metal cations are present and cause the As(V) to co-precipitate with or absorb into, iron oxyhydroxides. Whereas under reducing conditions, arsenite dominates and can be absorbed, co-precipitated with Fe or precipitate as metal sulfides (Heinrich-Salmeron *et al.* 2011). Arsenic mobility is also affected by pH and it increases as pH increases. Besides geochemical principals that govern As speciation in treatment systems, arsenic oxyanions and microorganisms interact and this also has a great role in arsenic transformation.

Organisms associated with arsenic are taxonomically diverse and metabolically versatile. They can be categorized into four groups; arsenate-resistance microbes, dissimilatory arsenate-respiring prokaryotes, chemoautotrophic arsenite oxiders and heterotrophic arsenite oxidizers. As-resistant microbes that carry the *ars* operon, which aerobically reduce As(V) to As(III), are widely distributed and have been studied extensively. In the second group, the microorganisms actively respire on As(V) under anaerobic conditions by utilizing arsenate as terminal electron acceptor with the respiratory electron transport chains. In fact, the respiration process can be coupled with the oxidation of organic matter such as acetate, formate, pyruvate, citrate and many other simple organics, which are notably present in passive treatment systems as intermediates in metabolic cycles. In chemoautotrophic arsenite oxiders, coupling of oxidation of As(III) and reduction of oxygen or nitrate (electron acceptors) can lead to CO_2 fixation into organic cellular material, while in heterotrophic arsenite oxidizers, heterotrophic oxidation occurs and As(III) is transformed into less toxic As(V) on the outer membrane of the cells (Han and Gu 2010). It should be noted that the above classification is on the basis of reducing /oxidizing capabilities of the microorganism

and other processes like methylation or adsorption to microbial biomass are also involved in arsenic detoxification and these mechanisms are present in passive treatment system.

A treatment system near the Teck Trail smelter in British Columbia, which treats seepage (Duncan 2010) containing elevated level of arsenic, cadmium and zinc, was selected for this study. Providing nutrients required to support the growth of arsenic active organisms, which are referred to as enrichment cultures, is one of the approaches that was applied. In functional sequence analysis, As resistant clones that showed growth in the presence of high arsenic concentrations were screened and identified. In parallel, sequence screening was performed on the short sequence section of the fosmids, referred to as fosmid-end sequences, in order to detect closest similarity matches with arsenic reference genes from a data base. Isolating and discovering As resistant microbes and genes involved in arsenic detoxification may lead to new and improved processes for As decontamination at mine sites.

Material and Methods

Bacterial enrichment cultivation and pyrosequensing

Samples were collected from six different positions in an anaerobic bioreactor and transferred to two different media. The arsenite oxidizing bacteria minimal salt enrichment medium described by Santini (Santini *et al.* 2000) was used with the addition of 5mM sodium arsenite ($NaAsO_2$), Na_2SO_4 0.07g/L and KNO_3 5g/L. The other anoxic minimal salts medium was used to enrich arsenic reducing bacteria (Zhang *et al.* 2008) containing 5mM sodium arsenate ($HAsNa_2O_4.7H_2O$) and lactate 1.121 g/L.

After 12 days incubation under anoxic conditions and several passages, a stable enrichment culture was obtained and, by using florescence staining with Sybergreen combined with flow cytometry, cultures with high numbers of cell counts were selected for DNA extraction and sequencing. PowerMax Soil DNA isolation kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA) was used for this purpose. DNA was PCR amplified using primers specific for the V6 variable region of the SSU rRNA gene: forward primer 454T-FB 5'□CTATGCGCCTTGCCAGCCCGCTCAG and reverse primer 5'□CGTATCGCCTCCCTCGCGCCATCAG. Attached also to the primers were barcodes and linker-adapters for the LibA chemistry used for sequencing. The resulting so-called pyrotags were sequenced by Centre d'innovation Génome Québec (Quebec, Quebec) and Université McGill (Montreal, Quebec). In total 19 samples were sequenced to empirically estimate their bacterial species richness. Eventually all the sequences were analyzed and compared using the QIIME pipeline (Caporaso *et al.* 2010).

Arsenic speciation was measured for cultures during each passage using high performance liquid chromatography (HPLC, Waters 1525 binary HPLC pump) and ultraviolet (UV) detection (Waters 2487 dual lambda absorbance detector). Detection at 191 nm was used to determine As(III) and As(V) concentrations in these cultures, which helped detect changes from initial concentrations. The mobile phase used was 0.01M sodium phosphate buffer with pH 6.0. The flow rate was 3 ml/min and the injection volume was 200 µl (Jedynak *et al.* 2008).

Functional metagenomic analysis

A total of three fosmid libraries were made from three separate samples taken from the Trail treatment system. Fosmids are circular pieces of DNA that are used to insert environmental DNA into E. coli clones. Details of the construction of these libraries can be found in (Taupp *et al*. 2009). These fosmids can be used to get functional information about the microbial community. As Figure 1 shows, two different approaches were used to identify arsenic detoxifying genes from a metagenomic library that was constructed from Trail passive treatment system samples. Determination of minimum inhibitory concentration of arsenic for *E.coli* was the first step prior to screening clone libraries and concentrations of 5.5 mM of As(III) and 130 mM of As(V) were used to select resistant arsenic fosmid clones. Using a Q-Soft XP gridding robot, fosmids were inoculated into agar in an extra large (XL) square biodish and incubated for a week. After the recovery of the positive growth fosmid clones, colony growth was

investigated with a photometric method using an optical density (OD) of 600 nm and the specific growth rate during exponential phase was calculated. In Figure 1, one example of a fosmid clone growth curve shows variable growth rates at different As(V) concentrations. Based on these growth curves, several colonies were chosen for fosmid purification with the FosmidMAX™ DNA purification kit (Epicentre, Madison, WI, U.S.A.). Fosmid DNA quality and yield were assessed by gel electrophoresis, fluorimetry and Bam H1 restriction enzyme digestion.

A database of arsenic resistant genes that were indentified in previous studies in the literature (a total of 384 sequences) (Stolz *et al.* 2006; Muller *et al.* 2007; Muller *et al.* 2007; Achour-Rokbani *et al.* 2010; Han and Gu 2010; Heinrich-Salmeron *et al.* 2011) was created. Then each of the fosmid-end sequences was queried against this database using the tblastn program included in the NCBI blastall v.2.2.20 package. For each sequence comparison, the best hit was picked based on the lowest Expect value (E-value), considering only those hits below a cutoff E-value threshold of 1E-6 and with amino acid identity scores of more that 30%. The E-value is a parameter that describes the number of hits one can expect to see by chance when searching a database of a particular size. The lower the E-value the more significant the match between the two translated proteins (http://www.ncbi.nlm.nih.gov/blast).

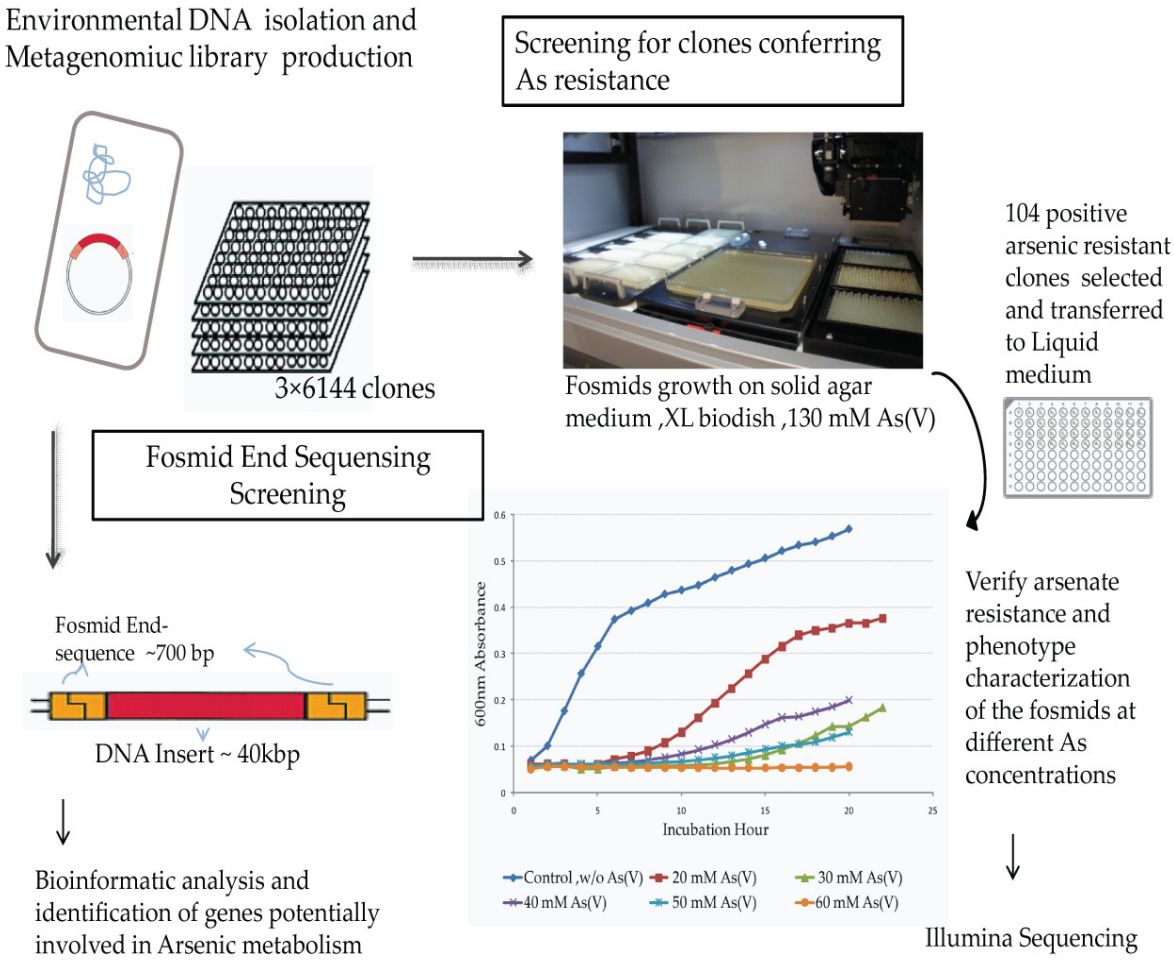

Figure 1. Overview schematic of methodology used to identify functional arsenic genes and hypothetical genes involved in arsenic metabolism

Results and Discussion

Enrichment culture characterization: Taxonomic profile

Taxonomic analysis showed that the microbes in all 19 enrichment cultures belonged primarily to five phylogenetic groups; *Firmicutes*, *Euryarchaeota*, *Bacteroidetes*, *Proteobacteria* and *Spirocheates*. Typically, in a phylogenetic analysis the sequences are clustered into what are called operational taxonomic units (OTU). Each OTU contains sequences that are more than 97% similar to each other and each OTU can be considered as a separate species. All of the sequences from enrichment samples were clustered into OTUs by Qiime. This resulted in many OTUs and so as to study only the most prevalent ones, a cut off threshold of at least 500 sequences per OTU was picked, which resulted in those shown in Figure 2. Each OTU was named according to its taxonomic classification. OTU counts are colored based on the contribution of each OTU to the total OTU counts present in each sample. For example, dark blue means that there was a high percentage of OTUs in that sample.

In the As(V) reducing medium the dominant groups were *Sedimentibacter*, *Clostridiales* and *Desulfitobacterium* (Figure2). *Sedimentibacter,* formerly belonging to the cluster *Clostridia,* are strictly anaerobic and have been isolated from hydrocarbon contaminated sediments (Breitenstein *et al.* 2002). The *Clostridium* genus has been extensively recognized for arsenate reductase activity (Stolz *et al.* 2006), and notably As resistance genes were identified within this group through the fosmid end sequence screening approach taken during this study. However the fosmid As genes that were related to *Clostridium* species were arsenate reductase, which is not associated with the dissimilatory arsenate-respiring *arr*AB operon (Table 1). Another predominant group that was found in the As(V) enrichment cultures was a *Desulfitobacterium* related OTU. Species of this genus are very versatile microorganisms capable of using a variety of electron acceptors such as nitrate, sulphate and metals such as As(V), Fe(III), Se(VI) and Mn(IV). Moreover, metal or metalloid respiration in this genus has great implication for bioremediation of arsenious as well as chlorophenolic compounds. One study (Niggemyer *et al.* 2001) identified an As(V) and Fe(III) reducing bacterium from arsenic contaminated sediments that coupled oxidation of formate to the reduction of arsenate. The isolate was closely related to *Desulfitobacterium hafniense* DCB-2T and *Desulfitobacterium frappieri* PCP-1T.

In the As(III) oxidizing bacteria minimal salt enrichment medium the major groups were *Simplicispira*, *Rhodoferax*, *Rhizobium* and *Acidovorax*. *Simplicispira* spp. have been reported from industrial wastewater as well as activated sludge that performed enhanced biological phosphorus removal. This group are chemoorganoheterotrophs, facultative anaerobes and are capable of nitrate reduction (Grabovich *et al.* 2006). Arsenite oxidation is common in *Rhodoferax* spp. Remarkably *Agrobacterium tumefacience,* which has the arsenite oxidixing operon (*aox*AB), was found in our fosmid As gene screening. The genes identified were associated with a two-component regulatory system (*aox*RS), which regulates the arsenite oxidizing operon (Muller *et al.* 2007). Arsenite oxidase catabolic subunit has also been reported in *Rhizobium* sp. (Stolz *et al.* 2006) and this group was found to be predominant in most of the As(III) enrichment cultures used in this study, i.e samples AsIII_1p3, AsIII_3p3, AsIII_5p7, AsIII_1P2 , AsIII_2p3, AsIII_1p7, AsIII_5p3, (Figure 2). The final group, *Acidovorax* spp. are other arsenic resistant microorganisms that are also well-known for having an arsenic operon (Muller *et al.* 2007).

The taxonomic group *Methanocorpusculum* was found in both arsenic enrichment media. This genus belongs to the domain Archaea and its species contain pathways for arsenic detoxification (Anderson *et al.* 2009). Members of the genus *Methanocorpusculum* commonly are found in wastewater and sewage bioreactors and in landfills (Huang *et al.* 2002) .They are generally autotrophs, producing their cellular carbon from carbon dioxide, and they gain energy from methanogenesis. Methanogens occupy a large variety of anaerobic environments, such as passive treatment system, and are usually found associated with decaying organic matter.

The differences in diversity within groups of samples were also examined by Qiime. Principal Coordinate Analysis (PCoA) was performed on the samples in order to reflect the dissimilarity between the As(V) reducing and As(III) oxidizing groups. The 19 samples were clearly clustered into two groups based on their microbial community composition, and, as was expected each cluster was related to the species of arsenic used in the medium (Figure 3).

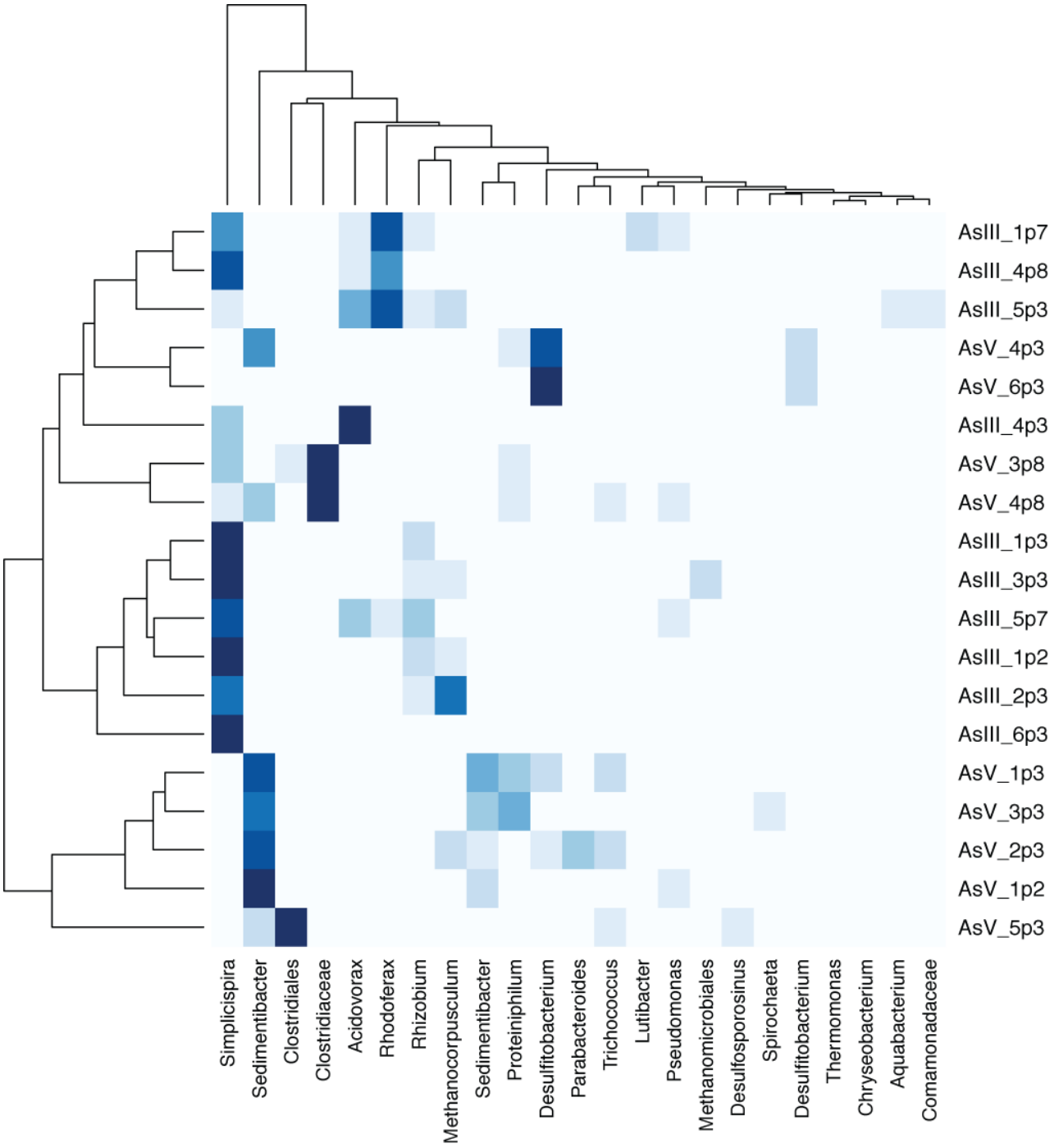

Figure 2. Taxonomic assignment of As(III) and As(V) enrichment cultures.

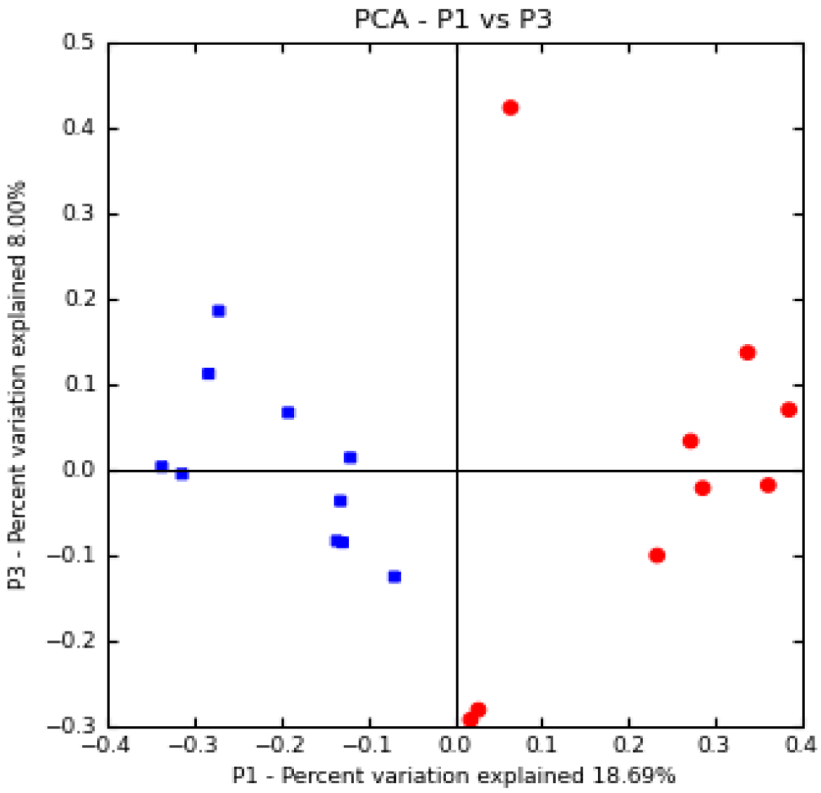

Figure 3. 2D-PCoA diversity analysis of enrichment cultures. As(III) oxidizing enrichment cultures and As(V) reducing enrichment cultures are represented respectively by blue and red points, respectively.

Enrichment culture characterization: Detection of arsenic transformation

As(III) and As(V) concentrations in the enrichment cultures were measured using HPLC-UV. Liquid samples were taken periodically for As speciation analysis and concentration measurements. The comparison of the results for As(V) enrichment medium showed noticeable transformation of these two As species. The different As peaks for sample As(V)-3P8, which was taken from a late passage of an arsenate reducing medium culture, is presented in Figure 4. By using the calibration plot and considering the dilution factors, As (V) concentration was calculated as 1.28 mM for this sample. The decrease of As (V) initial concentration from 5 mM to 1.28 mM is indicative of reduction of As(V) to As(III) (Figure 4c). This could be compared to the chromatograms for standard solutions of As(III) 18 ppm and As(V) 20 ppm with peaks at retention time of 0.988 minute and 6.918 minute, respectively (Figure 4a and b).The small peaks in the middle section of sample As(V)-3P8 (Figure 4c) may represent production of other arsenic intermediate compounds.

Screening results

In fosmid end sequence screening of the metagenomic library of the Trail passive treatment bioreactor, numerous genes potentially involved in arsenic responses were identified. Tables 1 and 2 display fosmid end sequences of the library and the homologous arsenic genes with the most closely related organism. The fosmid library contained 9558 end sequences with an average length of 700 bases each and all of these were compared, using the program tblastn, against the known arsenic resistance genes in the database. The genes that were identified as being present in the Trail fosmids were distributed in different process involved with arsenic biochemistry in the environment.

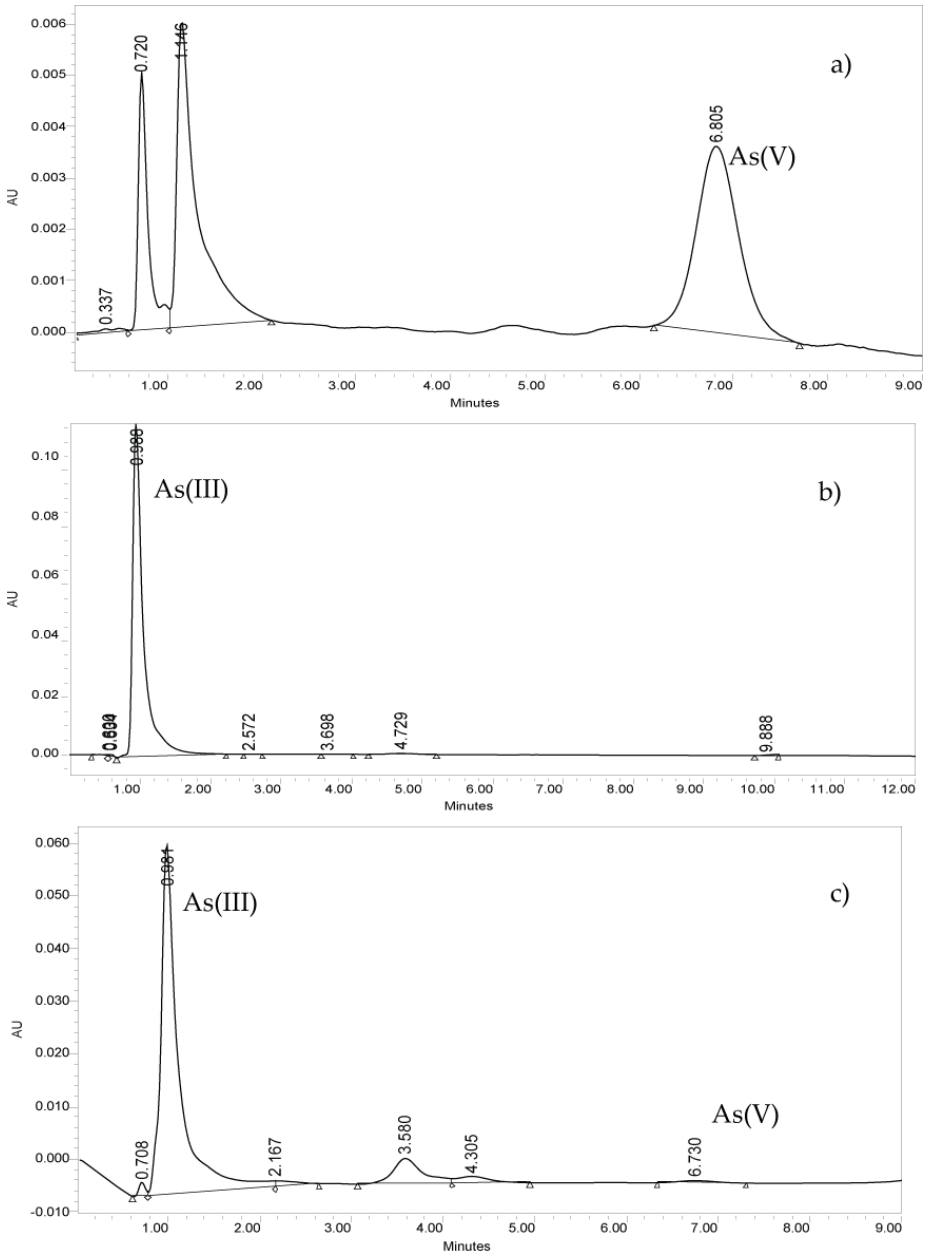

Figure 4. HPLC chromatographs of arsenic species of a) standard solution As(V) b) standard solution of As(III) and c) sample As(V)-3P8.

One important microbial resistance response to arsenic is bioaccumulation of arsenic and reduction of arsenate to arsenite by the *ars* operon, which was detected in our fosmid screening. In this process arsenic enters the cell through non-specific transporters like Pst and the arsenate is reduced to arsenite by ArsC, the cell then extrudes arsenite by the specific pump ArsB. Table1 shows the *arsC* genes related to two classes of tyrosin phosphatase and thioredoxin. Alignment best matches showed that *arsB* like sequences in the Trail fomids were related to the different species *Clostridium thermocellum*, *Herminiimonas arsenicoxydan*, and *Desulfovibrio desulfuricans*. ATPase and oxyanion transport ATP-binding protein, *pst* genes, were also detected in one of the fosmid end sequences, which is phylogenetically similar to *Alcaligenes faecalis* strain NCIB 8687. Apart from As resistance mechanisms, biotransformation is another microbial process that strongly influences the speciation and bioavailability of arsenic.

The biotransformation of arsenic, which results in the alteration of the redox state of arsenic, is another response towards arsenic and involves two different pathways. Dissimilatory arsenate-respiring organisms convert arsenate to arsenite via respiratory arsenate reductases, *arr* A/B operon, (Stolz *et al.* 2006; Han and Gu 2010). One example of an arsenate reducing microbe is *Shewanella* sp. ANA-3, and the *arrB* gene of this species was detected in one of the fosmids (Table 2). The other pathway involved in transformation of arsenic results in oxidation of arsenite to the less toxic arsenate and is associated with *aox* A/B operon, which is found in arsenite oxidizing bacteria (Stolz *et al.* 2006; Muller *et al.* 2007; Heinrich-Salmeron *et al.* 2011). One example of this mechanism was found in the fosmid sequences that contained the *aoxR* regulatory genes, and these were most closely related to *Rhodoferax ferrireducens*.

Inorganic arsenic can also be transformed into organic species in by methylation. Interestingly the As(III)-methyl-transferase gene (*arsM*) related to the *ars* RM-operon of *Rhodopseudomonas palustris* was similar to one of the fosmids. This indicates that the potential for the methylation process of arsenic detoxification is present in the Trail treatment system (Table 2) .

Conclusions

In this work, two different methods were used to identify arsenic resistance and detoxification mechanisms in the Trail treatment system, which treats seepage contaminated with arsenic and other metals, to investigate the possible mechanisms of As removal in this system. Using an As(V) medium we enriched *Sedimentibacter*, *Clostridiales* and *Desulfitobacterium* related organisms from the Trail site were enriched. The As(III) medium enriched a different group of microbes, namely, *Simplicispira*, *Rhodoferax*, *Rhizobium* and *Acidovorax.* In the other approach, a metagenomic library of genes from the Trail treatment system was screened against a database of As-related genes. Many As-related genes from the Trail fosmid library were found that were closely related to the microbes found in the enrichment cultures, including many other microorganisms. These genes are involved in all of the known arsenic resistance mechanisms showing that there are many ways in which the Trail site may be removing arsenic.

Table 1. Description of fosmid end sequences and their observed sequence similarities

Plasmid (insert size[bp])	Align. Length (aa)*	Closest similar As genes ([bp])	Organism	E value[1]	Identity (%)*	Function
FOS6247. CPF_F10 [808]	246	acr3-like (1041)	*Desulfovibrio desulfuricans* SSM1	1E-103	63 (115/246)	Arsenic efflux pump protein, ACR3 family
FOS6243. CPR_D16 [769]	222	aoxR (1284)	*Rhodoferax ferrireducens* T118	9E-65	48 (108/222)	AoxRS two-component regulatory system, associated with aoxAB operon (Muller *et al.*2007)
FOS6245. CPR_H06 [646]	170	arsT (987)	*Stenotrophomonas maltophilia* R551-3	3E-63	63.5 (108/170)	Thioredoxin reductase, related to thioredoxin (Trx) system within *ars* cluster (Rokbani *et al.*2009*)*
FOS6248. CPR_B12 [820]	186	aoxR (1365)	*Herminiimonas arsenicoxydans*	7E-62	46.24 (86/186)	AoxR regulatory protein
FOS6237. CPR_O13 [739]	178 70	pstB (886) phnC (831)	*Alcaligenes faecalis* strain NCIB 8687	3.00E-59 1.00E-16	58 (104/178) 55.7 (39/70)	ATPase and oxyanion transport ATP-binding protein that associate with *aox*AB operon , arsenite oxidation genes
FOS6243. CPR_D24 [769]	220	Nham_4423 (2454)	*Nitrobacter hamburgensis* X14 plasmid 2	5E-53	44 (97/220)	Heavy metal translocating P-type ATPase related to arsenate-resistance *ars* operon (Stolz *et al.*2006*)*

Footnotes for Table 1. * The Align. Length (aa) represents the sum of the non-overlapping alignment lengths for the gene of interest [1] The E-value is the highest E-values of all the aligned segments of the fosmid sequence with the gene of interest. [2] Identity (%) is the average amino acid homology of all the aligned segments between the fosmid and the gene of interest. Accession numbers for the arsenic resistance genes from NCBI are as follows: *acr3*-like (gi|291278433:1466871-1467911), *aoxR* (gi|89898822:3435400-3436683) *arsT* (gi|194363778:2224106-2225092), *aoxR* (gi|133737197:486934-488298), *pstB* (gb|AY297781.1|:40256-41141), *phnC* (gb|AY297781.1|:26667-27497), Nham_4423 gi|92109490:68287-70740) .

Table 2. Fosmid end sequences and their observed sequence similarities

Plasmid (insert size[bp])	Align. Length (aa)	Closest similar As genes ([bp])	Organism	E value*	Identity (%)*	Function
FOS6236. CPF_I16 [796]	115	arsB-like (1038)	*Clostridium thermocellum* ATCC 27405	1.00E-48	63.48 (73/115)	Arsenite pump
	117	acr3-like (1071)	*Herminiimonas arsenicoxydan*	7.00E-33	42.74 (50/117)	Arsenite pump
	122	arsC (411)	*Clostridium acetobutylicum*	2.00E-29	41.54 (27/65)	Arsenate reductase, tyrosine-phosphatase family
	96	arsC (408)	*Microbacterium* sp. A33	2.00E-19	45.8 (44/96)	Arsenate reductase
	87	arsRC(996)		3E.16	43.6 (38/87)	,thioredoxin family
FOS6240. CPF_K01 [809]	144	ORF5 (1377)	*Thiobacillus ferrooxidans*	6E-41	49 (71/144)	Signal recognition particle protein-like protein related to As operon
FOS6241. CPF_H12 [756]	163	arsM(852)	*Rhodopseudomons palustris* CGA009	2E-39	46.6 (76/163)	As(III)-methyl-transferase related to *ars*RM-operon
FOS6248. CPF_H18 [809]	140	arsR(921)	*Rhodococcus erythropolis* PR4 plasmid pREL1	3E-33	40 (56/140)	ArsR family transcriptional regulator
FOS6241. CPF_D08 [752]	202	TTHB129 (1221)	*Thermus thermophilus* HB8 plasmid pTT27	3E-31	35.6 (72/202)	Transposase attached to Aox AB operon and arsR (Muller *et al.*2007)
FOS6236. CPR_F17 [627]	104	arsC (417)	*Deferribacter desulfuricans* SSM1	8E-20	42.3 (44/104)	Arsenate reductase, glutaredoxin family
FOS6247. CPF_M19 [771]	134	arrB (705)	*Shewanella* sp. ANA-3	2E-23	44 (59/134)	Arsenate respiratory reductase (*arr*AB operon)(Stolz *et al.*2006)

Footnotes for Table 2. Accession number of arsenic resistance genes from NCBI are as follows: *arsB*-like (gi|125972525:1321854-1322891), *acr3*-like (1071)(gi|134093294:1980390-1981460), *arsC* -tyrosine-phosphatase family enzyme (411)(gb|AE001438.3|:111324-111734) *arsC* -thioredoxin family (gi|260161442:274-681) *arsRC* (gi|260161442:710-1705), ORF5 (gb|AF173880.1|:4348-5724), *arsM* (gi|39650317:187626-188477), *arsR* (gi|77454567:109131-110051),TTHB129 (gi|55978183:118438-119658), *arsC* -glutaredoxin family (gi|291278433:1466444-1466860) and *arrB* (gb|AY271310.1|:7167-7871).

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Acronyms

mM milimolar
nm nanometer
µl micro liter
pH hydrogen ion concentration
PCR polymerase chain reaction
rRNA ribosomal ribonucleic acid
DNA deoxyribonucleic acid