

EXECUTIVE SUMMARY

Background

Metals and metalloids are natural components of the earth's crust and cannot be degraded or destroyed. Activities such as mining expose metals and metalloids to the processes of weathering and oxidation, and to microbial activities. The principal consequence of these processes is the production of sulfuric acid and oxidized iron, the dominant soluble constituents of Acid Mine Drainage.

Fly Ash, siliceous particulate matter resulting from the burning of coal in coal-fired power stations, is typically disposed of in surface 'ash dumps'. Natural hydrological processes can mobilize potentially toxic elements in ash dumps into groundwater. Furthermore, there is current interest in utilizing Fly Ash for the neutralization of Acid Mine Drainage, thereby potentially simultaneously resolving two environmental problems. The 'Neutralization Solids' generated from the co-disposal process are generally rich in insoluble metals and metalloids (including potentially toxic elements such as As, Pb, Cr, etc.). Such solids have been considered for use in soil remediation, for back-filling or for other municipal and agricultural purposes.

We have suggested that 'Neutralization Solids', which may be sterile at the point of preparation, will inevitably develop microbial communities during exposure and maturation. We have proposed that such communities may include microbial species and activities which are potentially capable of deleterious changes. Potentially deleterious processes would include methanogenesis, sulphate reduction (sulphidogenesis) or metal/metalloid redox processes resulting in mobilization or enhanced toxicity.

Aims

One of the possible deleterious microbial processes which might occur in maturing 'Neutralization Solids' would be microbially mediated redox changes in metal and metalloids. As a model research program, we aimed to isolate microorganisms resistant to arsenic from matured AMD-FA neutralized solids, to characterize their arsenic resistance systems and to assess whether these organisms pose a potential 'threat' to the sustained use of 'Neutralization Solids'.

Results

This report describes the isolation and genetic characterization of arsenic-resistant microorganisms obtained from neutralised solids generated by emendation of fly ash [FA] with acid mine drainage [AMD]. Arsenic resistance strains of *Arthrobacter* and *Bacillus* were isolated from solid media containing 5% w/v sodium arsenate, inoculated with suspensions of FA-AMD neutralised solids. The phylogenetic assignment of the isolates was confirmed by analysis of the 16S rRNA gene sequences. Assays for arsenate reduction, using whole cell extracts, provided preliminary evidence for the presence of *arsC* gene products, a constituent of the *ars* operon. In order to identify and characterize the arsenic-resistance operons of the two isolates, genomic DNA from *Arthrobacter* and *Bacillus* was used to prepare gene banks in an arsenic sensitive mutant *E. coli* strain. Gene banks were screened for the presence of arsenic resistance (*ars*) genes. While *Arthrobacter* sp. *ars* genes could not be expressed in the mutant *E. coli* strain, the genes conferring arsenic resistance in the *Bacillus* sp. were cloned and sequenced. Homologues to the *arsR* (regulatory repressor), *arsB* (membrane located arsenite pump), *arsC* (arsenate reductase), *arsD* (second regulatory repressor/metallochaperone) and *arsA* (ATPase) genes from known arsenic resistance (*ars*) operons were identified. The *Bacillus* sp. arsenic resistance genes were shown to be arranged in an unusual manner with *arsB* immediately downstream of *arsR*. All *arsRBCDA* genes were transcribed in the same direction.

Analysis of the putative amino acid sequences of these *ars* genes revealed that the ArsR from *Bacillus* sp. was closely related to hypothetical proteins of other *Bacillus* species, whereas ArsB was closely related to the pump membrane protein of *S. aureus* plasmid p1258. The ArsC was closely related to ArsC of *Lysinibacillus* and was proposed to belong to the Trx clade of arsenate reductase due to the presence of cysteine residues which have been shown to play a catalytic role in reducing arsenate to arsenite in this Trx clade. The ArsD was closely related to the *Lysinibacillus* ArsD. The ArsA gene was closely related to *Lysinibacillus* ArsA and contained the Nucleotide Binding Motif (NBM), Metal Binding Motif (MBM) and the DTAP motif essential for its activity.

Conclusions and Project Outputs

We have demonstrated that arsenic resistance organisms are present in maturing FA-AMD neutralization solids, and have successfully isolated and identified the genes responsible.

The genes identified, although reasonably homologous to known *ars* resistance operon genes, show some peculiarities, particularly with respect to organization of the operon. This work therefore increases our understanding of the diversity of *ars* resistance genes and gene assembly.

These findings have some relevance to the possible *in situ* function of such organisms. The ArsC gene encodes a putative arsenate reductase, which is thought to function coordinately with an arsenite efflux system. We suggest that under anaerobic conditions, the natural functioning of this operon may have toxigenic capacity, i.e. to reduce low toxicity As^V (arsenate) to high toxicity As^{III} (arsenite) and to export the reduced metalloid to pore water where it could, potentially, be leached to groundwater.

Recommendations for Further Work

Several aspects of this research remain to be completed. Firstly, the functional contributions of the *ars* operon remain to be confirmed. This could be achieved at several levels: by northern blot analysis of mRNAs to confirm expression of the components of the operon; by demonstration of enhanced resistance to arsenic in the recombinant host strain; and by evidence of reduced resistance in the parent organism after inactivation or deletion of the genes and/or operon.

Secondly, analysis of the distribution and frequency of occurrence of homologous *ars* resistance genes in the FA-AMD neutralized solids metagenome should be assessed. This could be achieved by qualitative analysis of distribution through gene-specific PCR and phylogenetic analysis of the amplicons, and quantitatively via gene-specific qPCR.

Thirdly, *in situ* function should be determined. This might be achieved by a coordinated analysis of microbial function (demonstrating the capacity to reduce As^V *in situ*) and chemical determination of As speciation. Such studies could be confirmed by supplementation with organisms of known and demonstrated redox function.

