CHARACTERISATION OF THE ARSENIC RESISTANCE GENES IN *BACILLUS* SP. UWC ISOLATED FROM MATURING FLY ASH ACID MINE DRAINAGE NEUTRALISED SOLIDS

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ABSTRACT

An arsenic resistant *Bacillus* sp. UWC was isolated from fly ash acid mine drainage (FA-AMD) neutralised solids. A genomic library was prepared and screened in an arsenic sensitive mutant *Escherichia coli* strain for the presence of arsenic resistance (*ars*) genes. Sequence analysis of a clone conferring resistance to both sodium arsenite and sodium arsenate revealed homologues to the *arsR* (regulatory repressor), *arsB* (membrane located arsenite pump), *arsC* (arsenate reductase), *arsD* (second regulatory repressor and a metallochaperone) and *arsA* (ATPase) genes from known arsenic resistance operons. The *Bacillus* sp. UWC *arsRBCDA* genes were shown to be arranged in an unusual manner with the *arsDA* genes immediately downstream of *arsC*.

INTRODUCTION

Coal mining in South Africa is the second biggest mining sector after gold, with 16% export revenue and 4% of the gross domestic product. Acid mine drainage (AMD) and fly ash (FA) are produced through coal mining and, through the subsequent use of coal in power generation, contribute towards environmental pollution. AMD (pH 2.3 – pH 6.5) is formed in coal mining by the oxidation of metal sulphides found in the coal, rock and/or clay that is on top or below coal seams and dissolves heavy metals and metalloids (including arsenic) which pollute environmental waters. FA (pH 12) is a silicaceous particulate matter typically disposed of in surface 'ash dumps'. It consists mainly of silicon, aluminium, iron and calcium oxides. Arsenic can also be associated with FA, predominantly as arsenate,[1] usually complexed with carbonates or iron-manganese oxides.[2] As with AMD, natural hydrological processes can mobilise potentially toxic elements in ash dumps into groundwater. There is current interest in utilising FA for the neutralisation of AMD, thereby potentially simultaneously resolving two environmental problems.[3] The 'neutralisation solids' generated from the co-disposal process are generally rich in insoluble metals and metalloids (including potentially toxic elements such as arsenic, lead and chromium). Such solids have been considered for use in soil remediation, for back-filling and for other municipal and agricultural purposes.[4,5]

Current research in our laboratory has shown that 'neutralisation solids', which may be sterile at the point of preparation, develop microbial communities during exposure and maturation (Mutondo and Cowan, unpublished results). These communities may include microbial species whose activities could result, potentially, in environmental hazards. These transformations include methanogenesis, sulphate reduction (sulphidogenesis) or metal/metalloid redox processes, resulting in mobilisation or enhanced toxicity of metals and metalloids.

Arsenic is the most environmentally abundant toxic metal and all living organisms are exposed to low levels of arsenic naturally occurring in both water and soil.[6] In soils, arsenic is present in two oxidation states: As(III) (arsenite) and As(V) (arsenate).[7] Under oxidising conditions arsenate predominates and strongly adsorbs onto or forms minerals with iron, manganese and aluminium, thus reducing its solubility.[8] Generally, As(V) is the major species in arsenic-contaminated soils.[9] Under reducing or anaerobic conditions, the more toxic and soluble form of arsenic, arsenite, occurs. Due to the abundance of arsenic in the environment, microorganisms are under competitive pressure to maintain arsenic detoxification systems. These microbial activities are also responsible for arsenic cycling. It is possible that arsenic present in both AMD and FA could be mobilised into environments where neutralisation solids have been applied, increasing the risk of groundwater contamination.

The most common form of arsenic resistance in prokaryotes is through detoxification operons encoded on plasmids and/or genomes. These operons are diverse, but most often contain three genes: *arsR* (transcriptional repressor), *arsC* (arsenate reductase) and *arsB* (arsenite efflux membrane protein).[10] Some have two additional genes, *arsD* (second regulator and a metallochaperone) and *arsA* (ATPase), which confer higher resistance than the three-gene operon.[11] Arsenate entering the cell is reduced to the more toxic arsenite by ArsC, and is then transported out of the cell via ArsB (complexed with ArsA if it is present).[10] In this way, prokaryotes contribute to arsenite cycling in the environment.

In this study, we describe the isolation of an arsenic-resistant *Bacillus* strain from matured FA-AMD neutralised solids, together with the identification, cloning and characterisation of an *ars* operon.

MATERIALS AND METHODS

Bacterial strains, plasmids and media

Escherichia coli (*E. coli*) and *Bacillus* sp. UWC were grown at 37 °C in Luria-Bertani (LB) broth or agar medium,[12] with appropriate supplements of either ampicillin (100 µg/mL) or chloramphenicol (30 µg/mL) as required.

Isolation and identification of arsenic-resistant organisms

FA-AMD neutralised solids were obtained from Eskom[3] and dried. The solids were transferred into a pot (with a height of 25 cm, diameter of 20 cm, and a volume of 7 L) and mixed with moist garden soil (50% w/w) and exposed to the environment for six weeks prior to sampling. Samples (5 g) were suspended in 10 mL sterile water containing 2% (v/v) Tween 20,[13] then decanted into 2 mL sterile tubes before centrifugation at 5000 rpm for 90 s. Aliquots (200 µL) of the supernatant were aseptically spread in duplicate onto LB agar supplemented with 160 mM sodium arsenate, and incubated aerobically at 37 °C for 3–7 days. Colony morphology was observed under a stereomicroscope and Gram staining was carried out according to the method described by Cowan and Steel[14]. For identification of the arsenic-resistant isolate described in this study, a single colony was suspended in 50 µL water and treated for 5 min at 100 °C. This was used as a template for amplification using the universal bacterial 16S rRNA gene primers, E9F [9] and U1510R [15]. The reactions were carried out in an Applied Biosystems thermocycler in a 50 µL volume containing: 1x PCR Buffer; 100 ng DNA template; 0.5 µM of each primer; 200 µM of each dNTP and 1.25 U *Taq* DNA polymerase. The cycling conditions were: initial denaturation at 94 °C for 4 min; followed by 30 cycles of denaturation (94 °C for 30 s), annealing (52 °C for 30 s), elongation (72 °C for 2 min); and a final elongation step at 72 °C for 10 min. A partial (first 700 bp) 16S rRNA sequence was determined directly from the amplicon.

DNA isolation and manipulations

For the construction of the *Bacillus* sp. UWC genomic library in *E. coli* DH10B (Invitrogen), DNA was isolated from cells in late-stationary growth phase using the cetyltrimethylammonium bromide (CTAB) method[12]. Partially *Mbo*I-digested DNA was fractionated by electrophoresis on an agarose gel and those between 5 kb and 10 kb were ligated into the *Bgl*II restriction site of plasmid pEcoR252.[16] For routine cloning and manipulation procedures, standard procedures were used.[12] DNA fragments were purified from agarose gels using the PCR and Gel Band Purification kit (GE Healthcare, Cape Town, South Africa). Plasmid DNA was extracted using the Invisorb Spin Plasmid Mini Kit (Invitek, Berlin, Germany).

Screening for arsenic resistance genes

The *Bacillus* sp. UWC genomic library was screened in the *ars* deletion mutant *E. coli* ACSH50I[q 17] and clones were selected for their ability to complement the mutant on LB plates containing 1 mM sodium arsenate or 0.5 mM sodium arsenite. Plasmid DNA was extracted from positive clones and re-transformed to confirm arsenic resistance. Clones were analysed by restriction enzyme digestion and subcloned for sequencing.

Arsenic resistance assays

To test for the level of resistance conferred by the *Bacillus* sp. UWC *ars* genes, assays in *E. coli* ACSH50I[q] containing plasmids pArs8, pUM3 (as positive control)[18] and pEcoBlunt (as negative control)[19] were carried out in LB media containing chloramphenicol (30 µg/mL) and increasing concentrations of sodium arsenite or sodium arsenate. Overnight cultures were diluted 100-fold into fresh media and incubated at 37 ˚C for 5 h and absorbance was determined at 600 nm. The incubation period corresponded to the middle of the log phase of growth of a control culture grown under the same conditions. The resistance was expressed as a percentage of the optical density (OD_{600}) compared with the control culture with no added arsenic.

Sequencing analysis

Sequencing was by the dideoxy chain-termination method using an ABI 3130XL Genetic Analyser. Sequences were analysed using a variety of software program, including the DNAMAN package from Lynnon BioSoft. Comparison searches were performed using the gapped-BLAST program.[20] The *Bacillus* sp. UWC 16S rRNA and *arsRBCDA* gene cluster sequences have been deposited in the GenBank database with the following accession numbers: FJ554669 and FJ542199, respectively.

RESULTS

Isolation of an arsenic-resistant organism from maturing FA-AMD neutralisation solids

Several bacterial strains with different colony morphologies were isolated from FA-AMD samples on media containing 160 mM sodium arsenate. One of these, visualised as a Gram-positive rod, was analysed further. The 16S rRNA gene was amplified and partial sequencing (700 bp) indicated that it belonged to the genus *Bacillus* (accession number FJ554669), and was named *Bacillus* sp. UWC. This isolate was also able to grow in media containing 0.5 mM sodium arsenite.

Isolation of arsenic resistance genes

To identify and isolate *ars* genes, a genomic library of the *Bacillus* sp. UWC isolate was prepared and screened in the *E. coli* arsenic-sensitive mutant ACSH50I[q]. Approximately 6 000 transformed clones were plated on media containing 0.5 mM sodium arsenite, and 10 of these (pArs1-10) were arsenite resistant. The plasmids were analysed by restriction digestion (data not shown) and pArs8 was selected for further analysis. In order to identify the arsenic resistance genes in pArs8, several subclones were prepared and the plasmid inserts sequenced.

Sequence analysis of pArs8

The insert of plasmid pArs8 contained five open reading frames (ORFs) with high amino acid identity to genes associated with arsenic resistance genes in Gram-positive organisms (Table 1). The ORFs were encoded for ArsR (negative regulator)[21], ArsB (membrane pump for arsenite efflux),[22,23] ArsC (arsenate reductase),[24] ArsD (a second repressor and chaperone to deliver As(III) to ArsA)[25,26] and ArsA (an ATPase that associates with ArsB and links arsenite export to ATP hydrolysis)[22]. The highest sequence identities obtained were with *Bacillus* sp genes. BLAST analyses of the *Bacillus* sp. UWC *ars* gene products are shown in Table 1. The *ars* operon genes were positioned in the order *arsRBCDA* and transcribed in the same direction. While the *arsRBC* genes were ordered, as is commonly found in typical operons from the *E. coli* chromosome,[27] *Staphylococcus aureus* plasmid pI258 [29] and the *Bacillus* skin element[29] (Figure 1), the *arsD* and *arsA* genes were identified together, unusually situated downstream of the *arsRBC* genes. The *arsDA* genes are always associated and their linkage suggests that they have dependent functions in arsenic detoxification.[25] Operons containing *arsDA* confer high level of resistance to arsenic and have been described in *E. coli* plasmid R773,[30] *Acidithiobacillus caldus* transposon Tn*AtcArs* [20] and *Leptospirillum ferriphilum* transposon Tn*LfArs*.[31] In none of these operons were the *arsDA* genes directly downstream of the *arsRBC* genes. A BLAST search using the *Bacillus* sp. UWC *ars* gene sequences identified the presence of a similar *arsRBCDA* operon in several sequenced genomes

TABLE 1

Properties of the five arsenic genes isolated from *Bacillus* sp. UWC in plasmid pArs8

Gene	Size (aa)	Closest relationship based on sequence identity
arsR	104	69/80%, hypothetical protein, *Lysinibacillus sphaericus* C3-41
arsB	429	76/88%, YdfA, *Bacillus* sp. NRRL B-14911
arsC	141	77/88%, arsenate reductase (thioredoxin), *Geobacillus* sp. Y412MC10
arsD	119	48/72%, arsenical resistance operon repressor, *Bacillus* sp. MB24
arsA	586	74/85%, arsenical pump-driving ATPase (*Lysinibacillus sphaericus* C3-41)

Percentages are amino acid sequence identity/similarity.
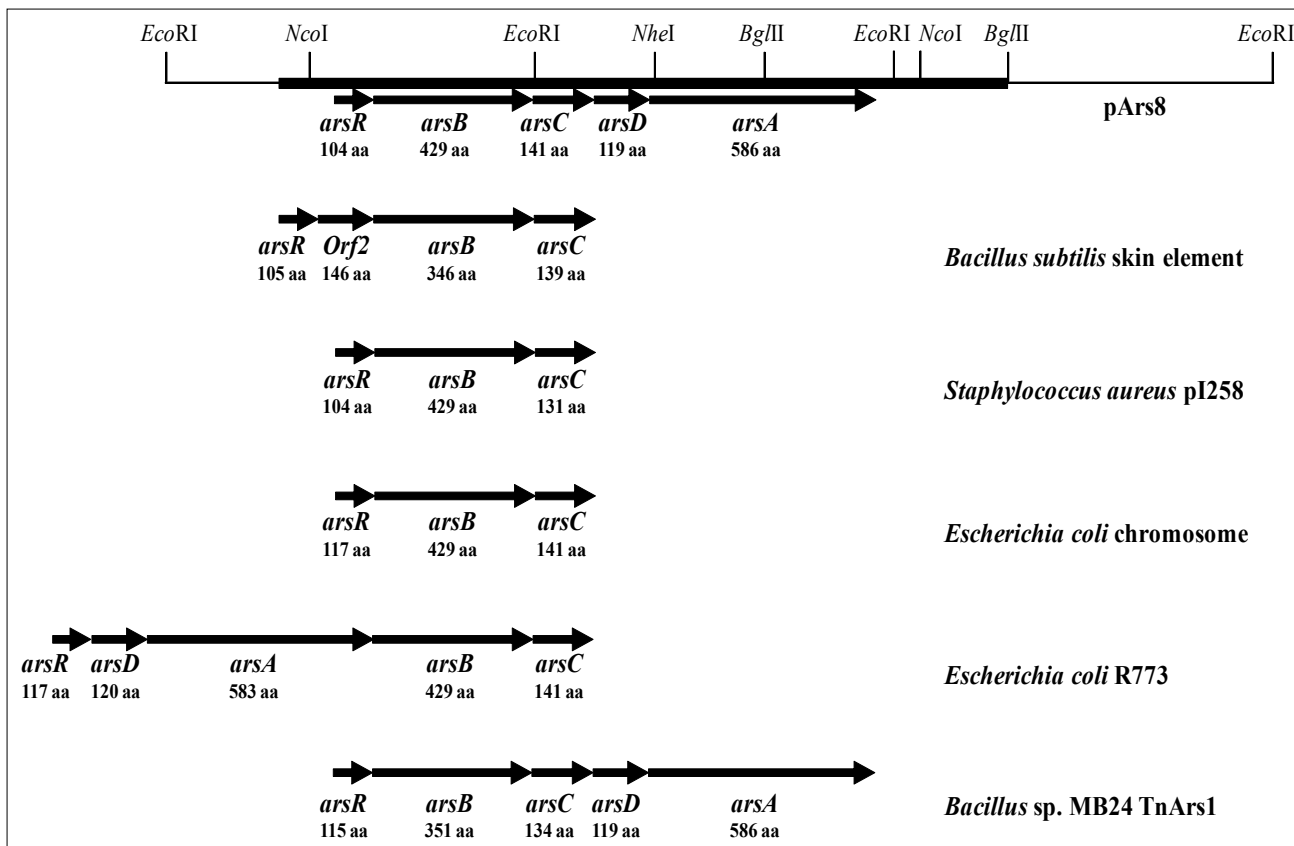
For comparison the *ars* operons from *E. coli* chromosome[27] and plasmid R773 [30], *Staphylococcus aureus* plasmid pI258 [28], *Bacillus subtilis* skin element[29] and the arsenic operon present in the TnArs1 transposon from a *Bacillus* sp. sequenced genome (accession number AY780525) are shown. As described in the text, several *arsRBCDA* operons were identified from sequenced genomes of *Bacillus cereus* and other *Bacillus* sp. The accession numbers are: NC_005707, NZ_ACMF01000073, NZ_ACLS501000058.1, NZ_ACLY010000133.1, NZ_ACMG01000030.1, NZ_ACMK010000128.1, NZ_ACML01000995.1, AF178758.

FIGURE 1
Diagrammatic representation of the ars operon isolated from *Bacillus* sp. UWC, showing the restriction map of pArs8 and the amino acid sizes of the gene products

from *Bacillus cereus* strains and other *Bacillus* species (Figure 1), suggesting a common organisation in this genus. However, the individual *ars* genes from *Bacillus* sp. UWC did not necessarily share the highest sequence identity with those identified from the sequenced genomes (Table 1). This suggests the possibility of a common ancestor operon, but with substantial evolutionary changes in the individual *ars* genes. The placement of the *arsDA* genes directly downstream of the *arsRBC* genes in this genus makes it tempting to speculate that the ancestor contained the typical *arsRBC* operon and later acquired the *arsDA* genes. In one *B. cereus* strain (ATCC 10987), the *ars* genes were identified on a plasmid (accession number NC_005707), while in the *Bacillus* sp. MB24 they occurred on a transposon. Typically,

ars operons containing *arsDA* genes are found on plasmids and transposons[19,30,31] and are therefore easily transferred by horizontal gene transfer.[31] However, since total genomic DNA was used to prepare the genebank from which the *Bacillus* sp. UWC *ars* genes were isolated, it is not possible to determine their location without further experimentation.

Analysis of the *arsRBCDA* gene products revealed the presence of motifs typical for these proteins. ArsR contained the typical ELCVCDL metal-binding[6,32] and the helix-turn-helix DNA binding[21,33] domains. Bacterial ArsCs have been divided into two families: those requiring thioredoxin[34,35] and those requiring glutathione/glutaredoxin.[36] Alignment of the
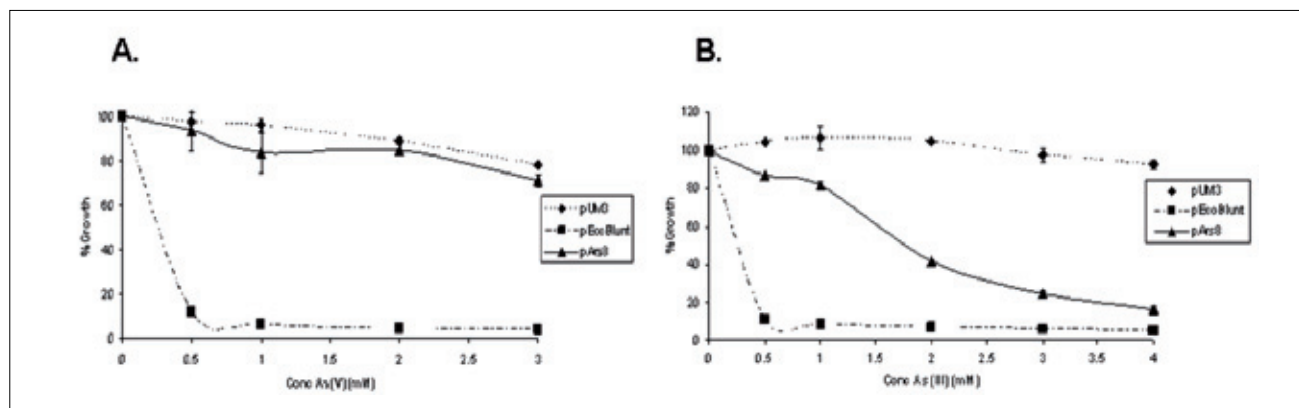
The positive (pUM3) (◆) and negative (pEcoBlunt) (■) controls are shown. The results represent the average duplicate experiments.

FIGURE 2
Resistance conferred in *E. coli* ACSH50Iq by the *Bacillus* sp. UWC arsRBCDA operon (pArs8) (▲) when grown in the presence of sodium arsenate (A) or sodium arsenite (B)

Bacillus sp. UWC ArsC with ArsC proteins from both groups suggests that it groups with the Trx clade and is therefore likely to be a thioredoxin type ArsC. Furthermore, it has the four conserved cysteine residues (Cys10, 15, 82, 89, numbering for the pI258 ArsC) identified as being required for activity in this family.[34,35] ArsD was originally reported to be a second, weak transcriptional regulator,[26] but has recently been shown to function as a chaperone, transferring arsenite to the ArsA for export by the ArsB and thereby conferring higher resistance.[25] The *E. coli* R773 plasmid ArsD has three vicinal cysteine pairs (Cys12-13, Cys112-113, and Cys119-120) and an additional cysteine, Cys18.[17,26] It has been proposed that only Cys12-13 and Cys18 are required for the metallochaperone activity,[37] while the Cys112-113 pair is associated with the weak transcriptional activity.[38] The *Bacillus* sp. UWC ArsD only contains Cys12-13 and Cys18, which might suggest that it functions only as the metallochaperone. The *Bacillus* sp. UWC ArsA contained the nucleotide binding domain and the conserved 12 residue motif (DTAPTGHTIRLL), called the DTAP motif,[39] in both the A1 and A2 halves. The three cysteine residues required for As(III)/Sb(III) binding and activation[40] are conserved in the *Bacillus* sp. UWC ArsA (Cys113, 172 and 422), suggesting that it too is involved in binding arsenite and possibly antimony.

Arsenic resistance in *E. coli*

When expressed in the *E. coli* *ars* mutant ACSH50I^q, construct pArs8 conferred an almost equal level of resistance to arsenate but a lower level of resistance to arsenite, compared to the positive control pUM3 (Figure 2). Although the experiment was not done in a low phosphate medium (phosphate competes with arsenate uptake thereby reducing the 'toxicity' of arsenate ions),[41] the comparative data with the arsenic-sensitive *E. coli* strain ACSH50I^q is strongly suggestive that the *ars* genes in pArs8 confer *in vivo* arsenic resistance.

DISCUSSION

To date, many arsenic-resistant microorganisms have been isolated from various environmental sources. Resistant isolates are found in both arsenic-uncontaminated[1] and arsenic-contaminated environmental samples.[42] Here, we report the isolation of a *Bacillus* isolated from FA-AMD neutralised solids which showed resistance to 160 mM sodium arsenate. Given the six week exposure of the FA-AMD sample to the environment, the chances of isolating common aerobic soil organisms were high[43] and several *Bacillus* species have been isolated from heavy metal-contaminated environments.[43,44,45]

General mechanisms of toxic metal resistance have been well studied and documented.[6,46] The most common resistance mechanism is the efflux of arsenite from the cell, encoded by either a three- or five-gene operon. The *Bacillus* sp. UWC isolate contains a five-gene operon, *arsRBCDA*, exhibiting a unique arrangement. While mRNA expression studies of the genes were not performed, the resistance conferred in *E. coli*, the presence of *arsDA* genes and the conserved active site amino acids identified in all the genes, together suggest that this operon is involved in resistance to high levels of arsenic in this *Bacillus* isolate.

These findings have some relevance to the possible *in situ* function of the *Bacillus* sp. UWC isolate (and other arsenic resistant organisms) in the FA-AMD neutralised solids. Although other factors such as pH and redox potential would also have an influence, the natural functioning of this operon may have toxigenic capacity: reducing low toxicity As(V) (arsenate) to high toxicity As(III) (arsenite) and exporting the reduced metalloid to pore water where it could, potentially, be leached to groundwater. To further evaluate this, analysis of the distribution and frequency of occurrence of homologous *ars* resistance genes in the FA-AMD neutralised solids metagenome should be assessed. This should be coordinated with an analysis

of microbial function (demonstrating the capacity to reduce As(V) *in situ*) and chemical determination of arsenic speciation.

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