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ORIGINAL PAPER

Extreme arsenic resistance by the acidophilic archaeon '*Ferroplasma acidarmanus*' Fer1

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Abstract '*Ferroplasma acidarmanus*' Fer1 is an arsenic-hypertolerant acidophilic archaeon isolated from the Iron Mountain mine, California; a site characterized by heavy metals contamination. The presence of up to 10 g arsenate per litre [As(V); 133 mM] did not significantly reduce growth yields, whereas between 5 and 10 g arsenite per litre [As(III); 67–133 mM] significantly reduced the yield. Previous bioinformatic analysis indicates that '*F. acidarmanus*' Fer1 has only two predicted genes involved in arsenic resistance and lacks a recognizable gene for an arsenate reductase. Biochemical analysis suggests that '*F. acidarmanus*' Fer1 does not reduce arsenate indicating that '*F. acidarmanus*' Fer1 has an alternative resistance mechanism to arsenate other than reduction to arsenite and efflux. Primer extension analysis of the putative *ars* transcriptional regulator (*arsR*) and efflux pump (*arsB*) demonstrated that these genes are co-transcribed, and expressed in response to arsenite, but not arsenate. Two-dimensional polyacrylamide gel electrophoresis analysis of '*F. acidarmanus*' Fer1 cells exposed to arsenite revealed enhanced expression of proteins associated with protein refolding, including the thermosome Group II HSP60 family chaperonin and HSP70 DnaK type heat shock proteins. This report represents the first molecular and proteomic study of arsenic resistance in an acidophilic archaeon.

Keywords *Ferroplasma* · Arsenic resistance · Arsenite · Proteomics · Primer extension

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Abbreviations

MSM	Mineral salts medium
RT-PCR	Reverse transcriptase polymerase chain reaction
2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis
MALDI-TOF	Matrix-assisted laser desorption ionization time-of-flight
ICP-MS	Inductively coupled plasma-mass spectrometry
HPLC	High pressure liquid chromatography
TCA	Tricarboxylic acid
acetyl-CoA	Acetyl-coenzyme A

Introduction

Arsenic is one of the most prevalent and toxic metals present in the environment (Meng et al. 2004). For example, the Iron Mountain site in California, USA, is one of the most acidic mine drainage sites, and contains total arsenic concentrations approaching the tenths of grams-per-litre (≤4.5 mM) range (Nordstrom and Alpers 1999; Edwards et al. 2000). Microorganisms inhabiting metal polluted environments encounter selective pressure to develop metal resistance mechanisms, and many acidophilic microorganisms are resistant to concentrations of toxic metals that exceed levels of resistance in neutrophilic microorganisms (Dopson et al. 2003). The genetic and biochemical mechanisms responsible for metal resistance in acidophilic archaea remain largely uncharacterized. This is despite the role of these organisms in bioleaching of ores for the extraction of metals (e.g. refractory gold present in arsenic-rich pyritic ores) and as intermediates in important biogeochemical cycles (Dopson et al. 2003).

The toxicity of arsenate [As(V); predominantly H_3AsO_4 at low pH] is due to its similarity to phosphate and it inhibits a large number of reactions, and in particular the production of ATP (Dopson et al. 2003). The toxicity of arsenite [As(III); $As(OH)_3$] is attributed to its strong affinity for sulfhydryl groups in proteins and is considerably more toxic than As(V) (Saha et al. 1999). In addition, As(III) depletes intracellular glutathione, resulting in oxidation of the cytosol and production of free radicals. In microorganisms, the most widely established arsenic resistance mechanism utilizes the *ars* operon [reviewed in Dopson et al. (2003) and Wong et al. (2004)], and these operons usually have three (*arsRBC*) or five (*arsRDABC*) genes (Rosen 1999). In general, the minimum set of genes for As(V) and As(III) resistance comprises *arsRBC* coding for a transcriptional regulator (*arsR*), a trans-membrane pump (*arsB*), and an As(V) reductase (*arsC*). In this system, ArsC mediates As(V) reduction to As(III) (Mukhopadhyay and Rosen 2002), which is then extruded from cells by the ArsB antiporter, catalyzing exchange of $As(OH)_3$ for protons (Meng et al. 2004). In addition, the anion-translocating ATPase ArsA increases the efficiency of ArsB, and ArsD is an arsenic metallochaperone that delivers As(III) to ArsA (Lin et al. 2006). Other genes associated with various *ars* operons include a putative thioredoxin reductase required for As(V) reduction with the reducing power from NADPH (*arsT*) (Wang et al. 2006), and two genes of unknown function that have weak homology to oxidoreductases (*arsO* and *arsH*) (Wang et al. 2006). One exception is *Sinorhizobium meliloti*, which lacks ArsB (Yang et al. 2005) and uses an aquaglyceroporin channel (AqpS) which facilitates efflux of As(III) generated internally by ArsC-catalyzed As(V) reduction. Thus, this organism is resistant to As(V) added to the growth medium but sensitive to As(III).

'*Ferroplasma acidarmanus*' Fer1 was isolated from the Iron Mountain site (Edwards et al. 2000; Dopson et al. 2004b) and grows in high concentrations of metals (Gihring et al. 2003; Dopson et al. 2004b; Baker-Austin et al. 2005). The 97% complete Fer1 genome sequence (http://www.genome.ornl.gov/microbial/faci/) includes a novel *ars* operon only containing *arsR* and *arsB* (Gihring et al. 2003). A further *Ferroplasma* lineage, termed *Ferroplasma* Type II, has been identified and its genome sequenced. Neither genome sequences contain a gene for an As(V) reductase, and along with the *Picrophilus torridus* genome (Fűtterer et al. 2004) constitute the first microorganisms identified without a recognizable *arsC* gene. The *P. torridus* genome contains the *arsRB* genes as well as a separately encoded *arsA* (Fűtterer et al. 2004). In addition, the circular plasmid pWCFS103 in *Lactobacillus plantarum* carries an arsenic resistance gene cluster that lacks *arsC* (van Kranenburg et al. 2005) although a chromosomal *arsC* is present (Kleerebezem et al. 2003). The minimal subset of *ars* genes present in '*F. acidarmanus*' Fer1 is surprising given the level of arsenic resistance in this isolate. *arsC* containing operons are present in other acidophiles, including *Acidithiobacillus ferrooxidans* (Butcher et al. 2000), *At. caldus* (Dopson et al. 2001; de Groot et al. 2003), *Acidiphilium multivorum* (Suzuki et al. 1998), and *Leptospirillium ferriphilum* (Tuffin et al. 2006).

In this study, we have investigated '*F. acidarmanus*' Fer1 arsenic resistance, which, along with *Halobacterium halobium* (Jones et al. 1991; Wang et al. 2004)

constitutes one of the first characterizations of archaeal arsenic resistance mechanism. This study suggests that '*F. acidarmanus*' Fer1 has an as yet unidentified alternative microbial arsenate detoxification mechanism that is apparently devoid of a functionally active means of reducing As(V).

Materials and methods

Strain and batch growth conditions

'*F. acidarmanus*' Fer1 (Edwards et al. 2000; Dopson et al. 2004b) was grown in shaken flasks (100 or 1,000 ml) in mineral salts medium (MSM) containing trace elements (Dopson and Lindström 1999), 20 g $FeSO_4{\cdot}7H_2O$ l^{-1}, and 0.04% (w/v) yeast extract. The basal salts were adjusted to pH 1.2 with H_2SO_4 and autoclaved. Growth experiments were carried out as previously described (Baker-Austin et al. 2005). Sterile yeast extract, trace elements, Fe(II), sodium hydrogen arsenate heptahydrate ($NaHAsO_4{\cdot}7H_2O$), and sodium (meta)arsenite ($NaAsO_2$) were added as required. Growth was measured as an increase in protein concentration after 96 h at 37°C (Dopson et al. 2004b, 2006). All experiments were carried out in triplicate and results presented as mean ± SD.

Transcriptional analysis of the *ars* operon

RNA was extracted from '*F. acidarmanus*' Fer1 cells using the RNAwiz kit (Ambion). The integrity and quantity of '*F. acidarmanus*' Fer1 RNA was determined using electrophoretic and spectrophotometric methods. Reverse transcriptase-PCR (RT-PCR) was carried out on *arsB* using specific primers (Fig. 1a; Table 1) and the access RT-PCR system (Promega) on approximately 1 µg '*F. acidarmanus*' Fer1 RNA. Primer extension analysis was carried out as previously described (Sawers and Bock 1989; Baker-Austin et al. 2005) using the primers ArsRBpe and arsR/Bperev (Table 1) which are complementary to the predicted *arsR* and *arsB* mRNA approximately 40 bases and 160 bases downstream from their respective 5′ ends. A plasmid containing the *arsRB* promoter region was used as template DNA for the corresponding manual DNA sequencing reactions. The '*F. acidarmanus*' Fer1 *arsRB* promoter region was isolated from '*F. acidarmanus*' Fer1 genomic DNA by PCR using primers arsR/BpeFor and arsR/BpeRev (Table 1) then cloned into PCR4-TOPO (invitrogen TOPO TA cloning kit) according to the manufacturer's instructions.

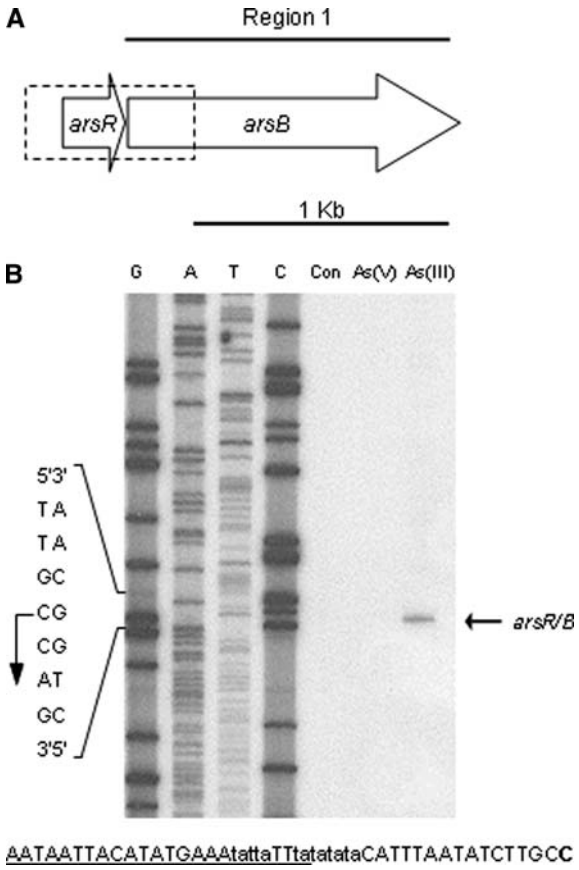

Fig. 1 Arrangement of the '*F. acidarmanus*' Fer1 *ars* operon showing the relative size and positions of the *arsRB* genes (**a**). *Dashed box* corresponds to the region amplified by PCR for cloning and *region 1* corresponds to the area targeted by RT-PCR (Table 1; the *size bar* is equivalent to 1 Kb). Primer extension analysis of the '*F. acidarmanus*' Fer1 *arsR/B* promoter region, showing corresponding sequence derived from sequencing gel, and cDNA transcripts derived from '*F. acidarmanus*' Fer1 mRNA (**b**). *Con* corresponds to '*F. acidarmanus*' Fer1 RNA derived from control cells (no arsenic addition), *As(III)* and *As(V)* corresponds to '*F. acidarmanus*' Fer1 RNA derived from cells cultured in 1 g l^{-1} As(III) and As(V), respectively. The transcriptional start site is indicated by a *bent arrow* at the *left* of the figure. The DNA fragment at the *foot* of this figure indicates putative TATA-box regions (*lower case*), the transcriptional start site (*final C*, *in bold*), and a putative imperfect 12-2-12 stem-loop is *underlined*

Two-dimensional polyacrylamide gel electrophoresis and mass spectrometry

For protein expression analyses, cells were grown to late log phase (96 h) in 1 l MSM with Fe(II) and yeast extract (as above) in the absence or presence of 1 g As(III) per litre. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was carried out according to Dopson et al. (2004a, 2006) that was originally adapted from Hesketh et al. (2002). Protein expression, spot matching, spot presence or absence, and statistics from the 2D-PAGE gels [number of replicates

Table 1 Primers used in this study

Primer name	Sequence (5′–3′)	Target region[a]
Cloning primers[b]		
arsR/Bpefor	CCGTGTATGTCAGATAATAC	Dashed box Fig. 1a
arsR/Bperev	GGCATTCCAGACTATATCCC	Dashed box Fig. 1a
RT-PCR primers[c]		
RNAarsBfor	ATGACATTATTATTTTACCTGG	Region 1 Fig. 1a
RNAarsBrev	TCAGACAATCAAAGACATTATG	Region 1 Fig. 1a
Primer extension primer[d]		
ArsR/Bpe	CCCTGATTTTTTCCATCTGG	arsR coding region
arsR/Bperev	GGCATTCCAGACTATATCCC	Predicted arsB coding region

[a] Regions on the '*F. acidarmanus*' Fer1 *ars* operon as shown in Fig. 1a

[b] Corresponds to the primer set used to amplify the putative *ars* promoter region

[c] Primers used to investigate transcription of *arsB*

[d] Designates the primer used for primer extension analysis of *arsR/B*

(n) = 2–3] were analyzed using ProteomWeaver version 1.3 (Definiens) (Dopson et al. 2005). Protein spots were excised from 2D-PAGE gels, trypsin digested, and analyzed using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (Hesketh et al. 2002). MALDI-TOF peptide mass fingerprint data was matched against the '*F. acidarmanus*' Fer1 genome sequence data using MASCOT (http://www.matrixscience.com) and MOWSE identification (Dopson et al. 2005, 2006).

Analysis of As(V) reduction

'*F. acidarmanus*' Fer1 cells were grown for 4 days and induced with 50 μM As(III) for 4 h. Cells were centrifuged at 10,000*g*, washed with MSM pH 1.2, and diluted tenfold in MSM. As(V) was added to a final concentration of 1 mM. The controls were MSM plus As(V); and non-induced cells plus As(V). Test samples were cells plus As(V); and cells plus 0.02% yeast extract plus As(V). Reactions were incubated for 18 h at 37°C with shaking. Aliquots of the supernatant were diluted with running buffer to appropriate levels for detection by inductively coupled plasma-mass spectrometry (ICP-MS) and injected into the high pressure liquid chromatography (HPLC)–ICP-MS. For some of the reactions, the cells were washed with MSM and lysed with water at pH 7.0. The supernatant from the cell lysate was diluted twofold with running buffer and injected into the HPLC–ICP-MS. Values were normalized to the highest value of the As(V) peak. No peaks were observed with buffer alone. The stock solution of As(V) was injected to test for natural reduction to As(III).

A Perkin-Elmer Sciex Elan 9000 ICP-MS with a cross flow nebulizer and Scott type spray chamber and cross flow nebulizer was used for arsenic determination. The operating parameters of the ICP-MS were optimized as follows: RF power, 1,000 W; plasma gas flow, 15 l min^{-1}; and nebulizer gas flow, 0.90 l min^{-1}. The optimum lens voltage was centred on rhodium sensitivity. Arsenic was detected using the peak-hopping mode of the ICP-MS at m/z of 75 with a dwell time of 300 msec. A Perkin–Elmer Series 200 pump and autosampler were used along with a Jupiter 300 C18 column in the HPLC separation of As(III) and As(V). The mobile phase consisted of 3 mM malonic acid, 5 mM tetrabutyl ammonium hydroxide, and 5% (v/v) methanol at pH 5.6 that was degassed and filtered and made with HPLC grade water (Sigma Aldrich) (Xiang et al. 2004). The arsenic species were separated under isocratic elution at a flow rate of 1.2 ml min^{-1}. After an injection of 20 μl of sample, all species eluted within 10 min. The position of each species was determined from the elution of As(V) and As(III) standards. A standard curve was calculated from 0.5, 1.0, and 5 μM mixtures of As(III) and As(V) in running buffer.

Measurement of the internal volume of '*F. acidarmanus*' Fer1

The internal volume of '*F. acidarmanus*' Fer1 was measured using five different concentrations of $^{3}H_2O$ and ^{14}C inulin (both GE Healthcare) each carried out in triplicate (Rottenberg 1979). The cell volume was calculated to be 1.64 ± 0.42 μl mg $protein^{-1}$ (n = 15; data not shown).

Results

Arsenic toxicity

To assess '*F. acidarmanus*' Fer1 arsenic resistance, batch toxicity culturing experiments were carried out.

Growth yields for '*F. acidarmanus*' Fer1 cultured in the presence of As(III) and As(V) were not significantly diminished until exposure to 5 g l^{-1} (67 mM) of either arsenical, suggesting extreme arsenic resistance (Fig. 2). Growth yields in 10 g l^{-1} (133 mM) of both arsenicals were significantly reduced compared to the no arsenic control, although '*F. acidarmanus*' Fer1 was more resistant to As(V) than As(III). After growth, cells cultured in the presence of 10 g As(III) l^{-1} were re-inoculated into MSM without arsenic and a similar level of growth and total protein was detected after 96 h (data not shown), indicating the viability of this culture.

Transcriptional analysis of the '*F. acidarmanus*' Fer1 *ars* operon

In this study, we have investigated regulation of the putative *arsR* and *arsB* genes in response to As(III) and As(V). As the stop codon of *arsR* overlaps the start codon of *arsB* (http://www.genome.ornl.gov/microbial/faci/) these genes are predicted to be translationally coupled and co-transcribed. To investigate whether the putative *arsRB* operon is co-transcribed and to locate the transcriptional start site(s), primer extension analysis was performed using RNA extracted from cells exposed to As(III), As(V), and control (no arsenic addition). When arsR/Bperev (located within arsB, Table 1) was used as a primer, only a single primer extension product was detected, and its position indicated that *arsRB* forms a single transcript when cells are grown in the presence of As(III) (Fig. 1b). The location of the *arsRB* transcriptional start site is 35 nucleotides downstream from the previously annotated ATG start of the *arsR* gene (Gihring et al. 2003). Hence, no cDNA product was generated from the primer extensions using the ArsR/Bpe primer (whose design was based on the published arsR annotation) as the transcriptional start site is located within this primer. Therefore, our results are consistent with the arsR gene being incorrectly annotated. No cDNA product was evident from primer extensions using RNA from the no arsenic control and As(V) exposed cells, suggesting that this operon is only induced by As(III) (Fig. 1b). This is consistent with results from *Escherichia coli* showing that the true inducer is As(III), and that ArsR does not recognize As(V) (Wu and Rosen 1993). The same result was obtained by RT-PCR analysis, as expression of arsB was only detected in response to As(III) (data not shown).

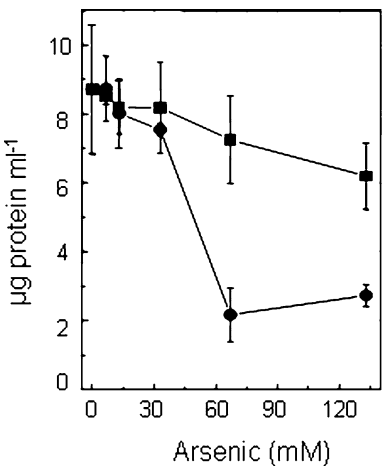

Fig. 2 Growth of '*F. acidarmanus*' Fer1 cells in the presence of As(V) (*filled squares*) and As(III) (*filled circles*). Growth was determined as protein increase after 96 h at 37°C. Data points are mean ± SD (n = 3)

Metal inducible operons in other organisms contain one or two imperfect '12-2-12' inverted repeats made up of two 12 bp partially palindromic DNA sequences separated by 2 bp that form a stem-loop structure near or overlapping the transcriptional start site of the metal regulated genes (Busenlehner et al. 2003). '*F. acidarmanus*' Fer1 arsRB also contains an imperfect 12-2-12 DNA sequence approximately 35 nucleotides upstream of this predicted transcriptional start site (Fig. 1b). Included in this region is a conserved archaeal TATA box 18 bp upstream of its transcriptional start site (Fig. 1b). Similar inverted repeats in a number of bacterial ars operon promoter regions have been shown to play an intrinsic role in allosteric regulation of repressor operator/promoter binding involved in transcription of these genes (Busenlehner et al. 2003). When examined in this context, the putative arsRB locus contains distinctive characteristics of a metal inducible operon.

Proteomic analysis of '*F. acidarmanus*' Fer1 exposed to As(III)

Protein spots identified as up-regulated ≥2.5-fold in the presence of As(III) (Fig. 3) were identified and analyzed (Table 2 and further details of protein identification are given in supplemental Table 1). Proteins up-regulated in the presence of 1 g As(III) per litre (13 mM) included α and β sub-units from the thermosome group II HSP60 family chaperonin and a DnaK type heat shock protein. These proteins play intrinsic roles in protein refolding. A further protein involved in repair of damage was the peroxiredoxin that scavenges for H_2O_2 in *E. coli* (Seaver and Imlay 2001). An increase in reactive oxygen species may have been as a

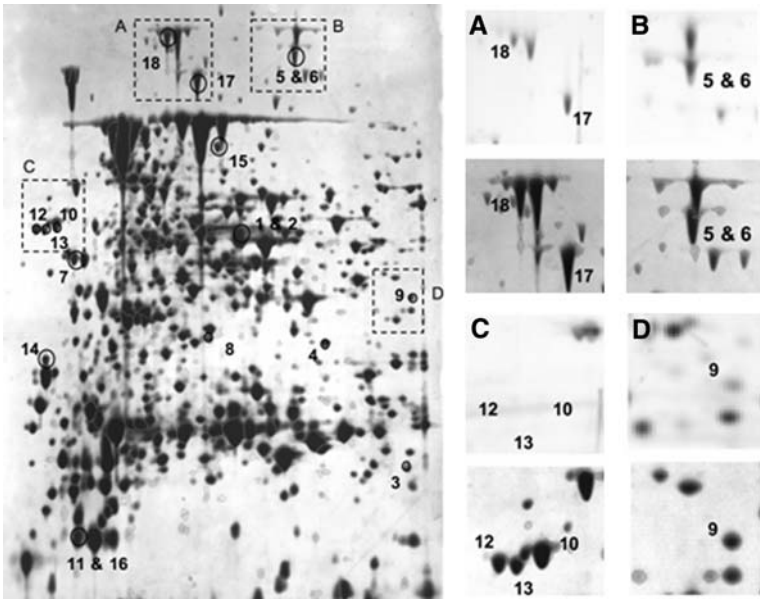

Fig. 3 Representative 2D-PAGE separation of '*F. acidarmanus*' Fer1 cells grown in the presence of 1 g As(III) per litre. *Circled spots* correspond to proteins ≥2.5-fold up-regulated in the presence of As(III) (spot numbers refer to Table 2). *Boxes A–D* highlight differences in protein expression in the absence (*upper panel*) and presence (*lower panel*) of As(III). Apparent spot intensity differences may not precisely match those listed in Table 2 (regulation), as the table values were derived by comparison of replicate 2D-PAGE gels (not shown)

result of the action of the 12.3-fold up-regulated NADH oxidase that produces H_2O_2 (Masullo et al. 1996) and has been shown to be induced by the presence of As(III) in humans (Lynne et al. 2000). Also, thioredoxin reductase that can either oxidize or reduce thioredoxin in a reversible reaction in *E. coli* was up-regulated (Williams 1995).

Other proteins identified as up-regulated in the presence of As(III) included enzymes feeding into, or part of, the tricarboxylic acid (TCA) cycle. These were components of pyruvate/2-oxoglutarate dehydrogenase complex and pyruvate ferreodoxin oxidoreductase, the product of both enzymes is acetyl-Coenzyme A (acetyl-CoA) that enters the TCA cycle, and the TCA cycle enzymes malate dehydrogenase and succinate dehydrogenase/fumarate reductase. In addition to central metabolic proteins, two ribosomal sub-units are also up-regulated suggesting protein synthesis may also be increased to produce the proteins required to combat the cellular stress caused by the addition of As(III).

Analysis of '*F. acidarmanus*' Fer1 As(V) reduction

HPLC–ICP-MS analysis of arsenic speciation in '*F. acidarmanus*' Fer1 cultured in the presence of As(V) was used to examine the ability of '*F. acidarmanus*' Fer1 to reduce As(V). Controls confirmed that As(V) is not abiotically reduced, which is consistent with previous data (Gihring et al. 2003). Analysis of the supernatant and '*F. acidarmanus*' Fer1 cytosol cultured in the presence of 1 mM As(V) revealed little detectable As(III) in the cytoplasmic extract (Fig. 4), and no changes in concentrations of As(V) in the growth media were detected. The amounts of As(V) and As(III) detected in either the cytoplasm or filtered culture media from '*F. acidarmanus*' Fer1 suggests that minimal As(V) reduction had occurred. Using our estimated '*F. acidarmanus*' Fer1 internal volume, the As(V) concentration inside of washed cells was calculated to be 6.3 mM compared to 1 mM in the medium. As washed and lysed cells were used, this suggests that As(V) accumulates inside '*F. acidarmanus*' Fer1 and that although no detectable As(V) reduction occurred the cells were resistant to As(V).

Discussion

A surprising aspect of '*F. acidarmanus*' Fer1 is the small number of recognized genes responsible for arsenic resistance given that it grows in 10 g l^{-1} As(III) or As(V) [133 mM; a tenfold higher concentration than previously reported (Gihring et al. 2003)], making it one of the most arsenic resistant species reported to date (Dopson et al. 2003). '*F. acidarmanus*' Fer1 resistance to As(III) appears to resemble the established *ars* operon system. RT-PCR and primer extension analyses suggest that expression of the *ars* operon is repressed in the absence of As(III). In the presence of As(III), *arsR* and *arsB* are co-transcribed, and transcription is most likely associated with active As(III) efflux. The identified *arsRB* operon contains the lowest number of functional

Table 2 Proteins ≥2.5-fold up-regulated in cells grown in the presence of 1 g As(III) per litre compared to controls in the absence of added As(III), as calculated from composite spot intensities from replicate 2D-PAGE gel sets

Spot[a]	Fer1 gene[b]	Putative protein function[c]	Regulation[d]
Metabolic and electron transport proteins			
1	168.1454	NADH oxidase	12.3
2	168.1428	Aspartate aminotransferase	12.3
3	164.1067	2-Keto-3-deoxy gluconate aldolase	11.9
4	164.1058	Malate dehydrogenase	3.9
5	164.1018	Pyruvate ferredoxin oxidoreductase (α sub-unit)	3.3
6	164.1059	Succinate dehydrogenase/fumarate reductase (flavoprotein subunit A)	3.3
7	162.916	Pyruvate/2-oxoglutarate dehydrogenase complex E1 component	2.9
8	148.237	Thioredoxin reductase TrxB	2.7
Biosynthetic proteins			
9	155.467	Cofactor biosynthesis protein	2.6
Transcription and translational components			
10*	168.1427	30S Ribosomal protein S2	NP[e]
11	160.772	50S ribosomal protein L7A	11.9
Stress proteins, chaperones and anti-oxidants			
12	165.1085	Thermosome β sub-unit (Group II HSP60 family chaperonin)	NP
13*	169.1708	HSP70 DnaK type heat shock protein	NP
14	158.616	Peroxiredoxin–Alkyl hydroperoxide reductase sub-unit F	4.4
15	157.567	Thermosome α sub-unit (Group II HSP60 family chaperonin)	2.5
Other proteins			
16	157.583	Hypothetical protein	11.3
17	168.1549	VCP-like ATPase	5.8
18	168.1549	VCP-like ATPase	2.6

Further details of protein identification are given in supplemental Table 1

[a] Refers to the spot numbers on Fig. 3. Proteins marked with an asterisk were identified as part of copper resistance (Baker-Austin et al. 2005)

[b] Contig and gene numbers for the protein as identified on the '*F. acidarmanus*' Fer1 draft genome analysis web page (http://www.genome.ornl.gov/microbial/faci/)

[c] Designates the identified protein name on the '*F. acidarmanus*' Fer1 draft genome analysis web page (http://www.genome.ornl.gov/microbial/faci/)

[d] Regulation, refers to the increase in protein expression determined by comparison of gel sets using ProteomWeaver

[e] NP designates the spot is not present in the corresponding gel set

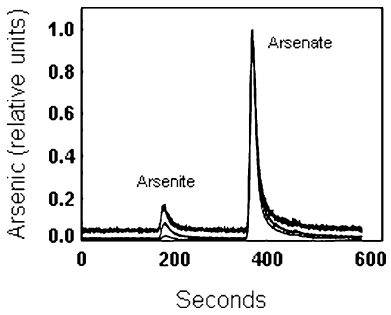

Fig. 4 Measurement of arsenic speciation to assay for As(V) reduction. The traces are filtered medium from As(III) induced '*F. acidarmanus*' Fer1 cells incubated in the presence 0.02% (w/v) yeast extract and 1 mM As(V) for 18 h at 37°C (*top trace*); control of medium plus As(V) in the absence of '*F. acidarmanus*' Fer1 cells (*middle trace*); and cytosol from '*F. acidarmanus*' Fer1 cells incubated in the presence of 0.02% (w/v) yeast extract and 1 mM As(V) (*bottom trace*)

genes for As(III) resistance reported in any microorganism. Determination of the '*F. acidarmanus*' Fer1 *arsRB* transcriptional start site by primer extension identified a loosely conserved region upstream of the transcriptional start site. This region contains core sequence structure, orientation, and a potential 12-2-12 inverted repeat consistent with bacterial operator/promoters regulated by members of the ArsR family (Busenlehner et al. 2003), and appears to promote expression in the presence of As(III) (Fig. 1b). In functionally characterized *arsR* systems such as *E. coli* (Xu et al. 1996), metal binding to the sensor protein (ArsR) weakens the DNA binding affinity, such that RNA polymerase can load and initiate transcription of the *ars* operon (Xu and Rosen 1999). There is no reason to suggest that the mechanism of *arsRB* tran-

scriptional regulation in '*F. acidarmanus*' Fer1 is atypical. However, the high levels of As(III) resistance demonstrated by this isolate coupled to the unusual metal-binding site at the C-terminal domain of this putative repressor (Gihring et al. 2003) remain to be investigated in greater depth.

Arsenic resistance in this isolate may be enhanced by expression and utilization of protein chaperones that refold proteins damaged by oxidative stress and non-specific inhibition of cytosolic enzymes by As(III), as has been recently suggested for copper (Baker-Austin et al. 2005). Metabolic and electron transport proteins up-regulated in response to As(III) probably reflect an increased bioenergetic demand conferred by arsenic. The proteomic response was supported by oxygen electrode experiments that revealed increased biotic Fe(II) oxidation when cells were exposed to 10 g l^{-1} (133 mM) As(V) or As(III) (Baker-Austin 2005). The broad functional spectrum of up-regulated enzymes is consistent with other proteomic reports concerned with metal resistance in this isolate (Baker-Austin et al. 2005) and other prokaryotes (Vido et al. 2001; Noel-Georis 2004) and suggests a whole cell protein and respiratory-wide response to metal stress.

Although the lack of evidence for a function does not preclude that it occurs, data from this study and Gihring et al. (2003) suggest that '*F. acidarmanus*' Fer1 is incapable of As(V) reduction. Also, neither of the sequenced *Ferroplasma* genomes (as well as *P. torridus*) contains a recognized *arsC* homologue. In *E. coli*, As(V) accumulates within the cells via active uptake by the phosphate uptake system (Elvin et al. 1987; Surin et al. 1987) and we have demonstrated that As(V) accumulates inside '*F. acidarmanus*' Fer1. Since As(V) must first be reduced to As(III) before it is extruded by ArsB (Liu and Rosen 1997; Mukhopadhyay and Rosen 2002; Jackson and Dugas 2003), an unknown, novel As(V) resistance mechanism is likely to be present in '*F. acidarmanus*' Fer1. Possible modes of arsenate resistance include the direct efflux of As(V), intracellular binding by an unknown chaperone, adaptation of the cellular proteins to be resistant to As(V), or due to the build up of high intracellular concentrations of phosphate. These hypotheses will be examined in future studies.

Proteomic analysis of As(III) did not detect increased expression of the ArsB pump. This is a membrane protein which are often highly insoluble and therefore, not readily resolved by 2D-PAGE. Experiments to over-express the '*F. acidarmanus*' Fer1 ArsB protein in *E. coli* such that it would be visible on a 1D-PAGE gel were not successful (data not shown). This was consistent with previous attempts to over-express ArsB in *E. coli* that were unsuccessful due to production of the ArsB protein being limited (Owolabi and Rosen 1990). The *arsA* homologue identified on the genome annotation has not been included in Fig. 1a. Also, it would not be expected to be identified by our proteomic study as it lacks both a promoter and a N-terminal domain and is likely to be a pseudogene. It may have been expected that ArsR would be identified as up-regulated however, its small size would have made it difficult to detect on PAGE gels.

In conclusion, we have initially characterized transcriptional and proteomic aspects of arsenic resistance by '*F. acidarmanus*' Fer1. The data suggest that As(III) resistance occurs by established mechanisms, but no evidence was found to suggest that '*F. acidarmanus*' Fer1 reduces As(V), suggesting a novel means of conferring As(V) resistance. Proteomic analysis of '*F. acidarmanus*' Fer1 suggests that a number of protein repair and modification enzymes were up-regulated in response to As(III).

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