

Arsenate reduction and expression of multiple chromosomal *ars* operons in *Geobacillus kaustophilus* A1

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Geobacillus kaustophilus strain A1 was previously isolated from a geothermal environment for its ability to grow in the presence of high arsenate levels. In this study, the molecular mechanisms of arsenate resistance of the strain were investigated. As(V) was reduced to As(III), as shown by HPLC analysis. Consistent with the observation that the micro-organism is not capable of anaerobic growth, no respiratory arsenate reductases were identified. Using specific PCR primers based on the genome sequence of *G. kaustophilus* HTA426, three unlinked genes encoding detoxifying arsenate reductases were detected in strain A1. These genes were designated *arsC1*, *arsC2* and *arsC3*. While *arsC3* is a monocistronic locus, sequencing of the regions flanking *arsC1* and *arsC2* revealed the presence of additional genes encoding a putative arsenite transporter and an ArsR-like regulator upstream of each arsenate reductase, indicating the presence of sequences with putative roles in As(V) reduction, As(III) export and arsenic-responsive regulation. RT-PCR demonstrated that both sets of genes were co-transcribed. Furthermore, *arsC1* and *arsC2*, monitored by quantitative real-time RT-PCR, were upregulated in response to As(V), while *arsC3* was constitutively expressed at a low level. A mechanism for regulation of As(V) detoxification by *Geobacillus* that is both consistent with our findings and relevant to the biogeochemical cycle of arsenic and its mobility in the environment is proposed.

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INTRODUCTION

Micro-organisms have an important impact on the biogeochemical transformations of arsenic, and their activities affect the mobility and toxicity of this element. Elevated amounts of arsenic can occur, especially in geothermal environments (Stauffer & Thompson, 1984). Thus, thermophilic micro-organisms such as *Geobacillus* species that thrive in geothermal soils and sediments are of particular interest for studying the mechanisms for the detoxification of arsenic compounds.

Genes for arsenic detoxification were first discovered and characterized in Gram-negative bacteria (Chen *et al.*, 1986; Rosen, 1999). These genes are often plasmid-encoded and are widespread in prokaryotes (Bruhn *et al.*, 1996; Ji & Silver, 1992b; Mukhopadhyay & Rosen, 2002; Mukhopadhyay *et al.*, 2002; Oden *et al.*, 1994; Rosenstein *et al.*, 1992). In thoroughly studied systems, the *ars* operon has been reported to contain the five genes *arsRDABC*, as in *Escherichia coli* (Cai & DuBow, 1996; Rosen *et al.*, 1992), or at least the three genes *arsRBC*, as in *Staphylococcus aureus* (Silver *et al.*, 1993). ArsA and ArsB are components of an arsenite-transporting ATPase, where ArsA is the ATPase and ArsB is the transmembrane component of the complex. In micro-organisms in which ArsA is absent, ArsB acts as a single-component transporter. Both ArsA and ArsB may confer resistance to arsenite and antimonite (Rosen, 1999). In its dimeric form, ArsR is an arsenite-responsive repressor that binds to the *ars* promoter (Xu & Rosen, 1997). ArsC is the arsenate reductase that converts arsenate to arsenite, conferring resistance to arsenate (Martin *et al.*, 2001). More recently, new arrangements of arsenic-resistance genes have been discovered in a

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Abbreviations: LMWP family, low-molecular-weight phosphatase family; qRT-PCR, quantitative real-time PCR.

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Two supplementary figures and a supplementary table are available with the online version of this paper.

number of micro-organisms, including Bacteria (Achour-Rokbani *et al.*, 2010; Branco *et al.*, 2008; Li & Krumholz, 2007; Murphy & Saltikov, 2009; Ordóñez *et al.*, 2005) and Archaea (Gihring *et al.*, 2003; Wang *et al.*, 2004).

Arsenic resistance and its genetic determinants have not been investigated before in *Geobacillus*. Among the closest relatives of *Geobacillus*, arsenic-resistant members of the genus *Bacillus* have been described that are capable of tolerating arsenate levels up to 20 mM (*Bacillus indicus*, *Bacillus arsenicus*) (Shivaji *et al.*, 2005), but the genetic determinants and molecular bases of arsenic resistance in these species are unknown. Furthermore, a number of *Bacillus* species, including *Bacillus arsenicoselenatis*, *Bacillus selenitireducens* (Switzer Blum *et al.*, 1998) and strains isolated from arsenic-rich environments (Fisher & Hollibaugh, 2008; Santini *et al.*, 2004), have been reported to use As(V) as the terminal electron acceptor. Such species rely on respiratory arsenate reductases, a class of enzymes unrelated to detoxifying arsenate reductases.

Here we report the molecular characterization of arsenic resistance in *Geobacillus kaustophilus* strain A1, previously isolated from a geothermal soil on the Metallifere Hills (Italy) and able to tolerate up to 80 mM As(V) (Cuebas *et al.*, 2011). We have demonstrated arsenate reduction, identified the genes responsible for arsenic resistance, and monitored their expression in strain A1. Two arsenate reductases are induced in the presence of As(V), and a third one is constitutively expressed. Based on these observations, a role for these genes and their regulation in arsenic detoxification is proposed.

METHODS

Culture conditions. Cells were cultured at 55 °C in Luria-Bertani (LB) broth. Batch cultures were inoculated to obtain a density corresponding to OD₅₄₀ ~0.025, with aliquots withdrawn from mid-exponential phase (OD₅₄₀ 0.3–0.6) cultures. For quantification of gene expression, cell cultures were treated with 5 mM sodium arsenate during the exponential phase of growth. Growth was monitored at 540 nm on a Beckman DU-520 spectrophotometer (Beckman Coulter).

Analysis of As species. To monitor the concentration of As(V) and As(III) during growth, cells were cultured on minimal medium (MM) containing 10 mM ammonium sulfate, 2 mM potassium phosphate, 2 mM magnesium phosphate, 0.5 mM calcium chloride, 0.1 mM iron (III) chloride, 0.01 mM sodium tetraborate, 9 mM manganese (II) chloride, 0.75 μM zinc sulfate, 0.37 μM copper chloride, 0.125 μM sodium molybdate, 0.185 μM vanadyl (IV) sulfate and 0.065 μM cobalt sulfate, pH 6.5, supplemented with 0.2% sucrose as the carbon and energy source. The medium was amended with 20 mM As(V), and aliquots were collected every 2 h and filtered using a 0.45 μm pore-size nylon disposable filter to remove cells. The filtrate was then analysed by HPLC. Calibration standards were prepared using matrix-matching background solutions containing MM and known amounts of arsenate and arsenite. MM containing 20 mM As(V) without cells was used as a control. Measurements of cellular arsenic uptake were conducted by growing the cultures on MM with 5 or 20 mM As(V) and collecting samples during the exponential phase of growth. Cells were centrifuged,

washed with MM and digested in ultrapure concentrated nitric acid for 20 h at room temperature. The total arsenic content was determined by inductively coupled plasma optical emission spectroscopy (ICP-OES). Cells grown in the absence of As(V) were used as a control. The concentrations of As(V) and As(III) were determined by HPLC. A high-performance liquid chromatograph (Shimadzu) was used to measure arsenite [As(III)] and arsenate [As(V)] concentrations. The instrument had a Hamilton PRP-X100 anion-exchange column and a UV absorbance detector set at 195 nm.

DNA isolation and sequence analysis. Genomic DNA was isolated as previously described (Cuebas *et al.*, 2011). Briefly, cell pellets were washed and resuspended in 10 mM Tris/HCl, pH 8.0, containing 20% (v/v) sucrose, and incubated with 2.5 mg lysozyme ml⁻¹ for 45 min at 37 °C, followed by a second incubation for 2 h at 55 °C with Tris-EDTA buffer, 10% SDS and proteinase K. Finally, the DNA was extracted with phenol/chloroform and treated with RNase A. Segments of *ars* operons were amplified using pairs of specific primers (Supplementary Table S1) in 25 μl PCR reactions assembled with iProof DNA polymerase (Bio-Rad). The strategy to design the primers is described in Results. PCR products were sequenced by GENEWIZ, and the sequences obtained in this way were assembled with BioEdit. For maximum-parsimony analysis, ArsC sequences from *G. kaustophilus* HTA426 (GK) and strain A1 were aligned to characterized and putative homologues of detoxifying arsenate reductases using CLUSTAL W (Larkin *et al.*, 2007). The alignment was resampled 100 times with Bootstrap, and analysed with Protpars, and a consensus tree was derived applying the program CONSENSE, included within the PHYLIP package (Felsenstein, 2004).

RNA isolation and expression analyses. Total RNA was isolated from exponentially growing cultures (OD₅₄₀ 0.3–0.6). Cells were mixed with two volumes of RNAProtect (Qiagen) and centrifuged at 10 000 r.p.m. for 1 min in an Eppendorf microcentrifuge (Brinkmann Instruments, Inc.). RNA was extracted from the cell pellets using the RNeasy Mini kit (Qiagen) and treated with DNase I (Ambion), as recommended by the manufacturers. Absence of contaminating DNA was confirmed by PCR using primers for amplification of the 16S rRNA gene. The quantity and quality of the RNA obtained were evaluated both spectrophotometrically on a NanoDrop ND-1000 spectrophotometer (NanoDrop) and by agarose gel electrophoresis (Sambrook *et al.*, 1989). For RT-PCR analysis, total RNA (0.5 μg) was reverse-transcribed, and the cDNA thus obtained was amplified by PCR in 25 μl reactions using the Enhanced Avian HS RT-PCR kit (Sigma-Aldrich). The amplification products were separated on a 1.2% agarose gel by electrophoresis, and the gel images were acquired using a Gel Logic 440 Imaging system (Eastman Kodak). Furthermore, absolute quantification of specific transcripts was performed by quantitative real-time PCR (qRT-PCR) using the iScript One-Step RT-PCR kit with SYBR Green (Bio-Rad) and the real-time detection system iCycler iQ (Bio-Rad). Reactions, in triplicate, were carried out according to the protocol of the manufacturer, and used 0.2 μg total RNA in a 25 μl reaction. Two negative controls were included, one without reverse transcriptase and one omitting the template. Primers were designed using the OligoPerfect Designer software (Invitrogen) to have a composition that was suitable for use in both RT-PCR and qRT-PCR (Supplementary Table S1). Standard curves were constructed for each of the three *arsC* genes using dilutions (range of standards: 10⁴–10⁹ copies μl⁻¹) of the corresponding PCR amplicons (Chini *et al.*, 2007; Mensink *et al.*, 1998). In parallel, the level of the *rpoB* transcript, detected using the primer pair QrpoB-F and QrpoB-R (Supplementary Table S1), was monitored as the internal standard to ensure that equal amounts of RNA were used in each reaction.

RESULTS

G. kaustophilus strain A1 reduces arsenate

To determine whether strain A1 assimilated arsenate during growth, cells were digested in nitric acid and the total arsenic accumulated was measured. The total amount of arsenic detected in cells grown in the presence of 20 mM arsenate (equivalent to 1.5 g arsenic l⁻¹) was approximately 4.4 g l⁻¹, which is three orders of magnitude lower than the external concentration. Thus, intracellular accumulation of arsenate was ruled out. Because arsenate is known to enter the cell through phosphate channels, we hypothesized that a mechanism for maintaining a low cytoplasmic As(V) was in place. Therefore, to determine whether strain A1 had the ability to reduce As(V) to As(III) and transport it outside the cell, we monitored both oxyanions in a time-course experiment. The results indicated that A1 is capable of reducing arsenate to arsenite during growth (Fig. 1). Degenerate primers targeted to the *arrA* genes (Pérez-Jiménez *et al.*, 2005) did not yield amplification (data not shown), and BLASTP similarity searches of the GK genome did not reveal any homologues of ArrA or ArrA-related sequences, e.g. ArxA (Zargar *et al.*, 2010), indicating that respiratory arsenate reductases are absent in strain A1 or highly divergent from known proteins. From this observation, in combination with the fact that *Geobacillus* A1 requires oxygen for growth (Cuebas *et al.*, 2011), it was postulated that the reduction of As(V) is most likely a process of detoxification.

Identification of the genetic determinants of As(V) resistance

Arsenic resistance is commonly mediated by detoxifying arsenate reductases. Since strain A1 is resistant to high As(V) and able to reduce it to As(III), we tested by PCR amplification whether the strain possesses an arsenate reductase. GK encodes two clusters of *ars* genes and a

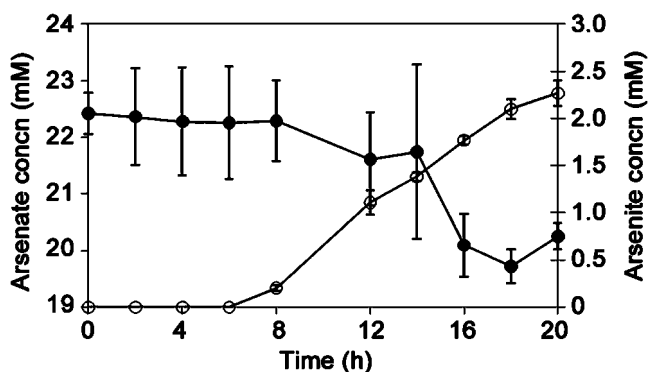


Fig. 1. Time-course analysis of arsenate transformation in MM. Arsenic species were measured by HPLC. ●, Arsenate concentration; ○, arsenite concentration. Experiments were performed in triplicate; error bars, SD.

monocistronic detoxifying arsenate reductase. It cannot be excluded that strain A1 possesses additional factors that contribute to arsenic resistance. However, given that both GK and A1 show the same level of arsenate resistance (Cuebas *et al.*, 2011), and no obvious additional sequences with roles in arsenic detoxification have been identified in the GK genome, we focused on the analysis of the putative *ars* sequences. Therefore, primers for the amplification of the two *ars* clusters and the monocistronic reductase were designed on the basis of the published GK genome (Takami *et al.*, 2004). To ensure reductase-specificity of the primers, the genome segments spanning from sequence GK3224 to GK3222, and from GK0587 to GK0589, were aligned using CLUSTAL W, and the most dissimilar regions between homologous sequences were selected as targets for the design of PCR primers. First, each pair of primers was tested on genomic DNA isolated from GK (Fig. 2a for location, Supplementary Table S1 for primer sequences). All pairs of primers produced amplicons and their specificity was confirmed by sequencing.

Then, the same pairs of primers were used for PCR amplifications using DNA isolated from strain A1 as template. In this way we obtained the sequences of the segments *ars1* and *ars2*, each of which included a partial *arsR* gene, a full *arsC* and a partial *arsB* gene (Fig. 2a). The nucleotide sequence of each fragment displayed a high degree of conservation with respect to homologous sequences in GK. In fact, the partial sequences of *ars1* and *ars2* were 91 and 98 % identical to the corresponding sequences of GK, respectively. We also obtained the partial nucleotide sequence of strain A1 *arsC3* (362 bp), which was 100 % identical to its GK homologue. At the protein level, the ArsB1 transporters and ArsC1 reductases of GK and strain A1 were 94 and 95 % identical (97 and 98 % similar), respectively, while the ArsB2 pumps and ArsC2 reductases were both 99 % identical (99 and 100 % similar, respectively) to their GK counterparts.

Sequence analysis of arsenate reductases

Two clusters of genes related to arsenate detoxification are found at three distant locations within the GK chromosome, and each includes a transcriptional regulator, an arsenite pump and an arsenate reductase. Both sets of genes display a high level of similarity to one another, suggesting that they might be the result of a duplication event. The proteins encoded by GK3222 (ArsC1, 139 aa) and GK0589 (ArsC2, 140 aa) are >91 % identical (99 % similar) to each other, and fall in a well-defined clade that comprises arsenate reductases belonging to the low-molecular-weight phosphatase (LMWP) family (Fig. 3). The representative member of this type of reductase was originally characterized in *S. aureus* (Ji & Silver, 1992a). This family of proteins is characterized by the presence of the tyrosine-specific protein phosphatase (PTP) signature motif CX₅R, also known as a P-loop (Jackson & Denu, 2001), located in the N-terminal region and containing the active site. Arsenate

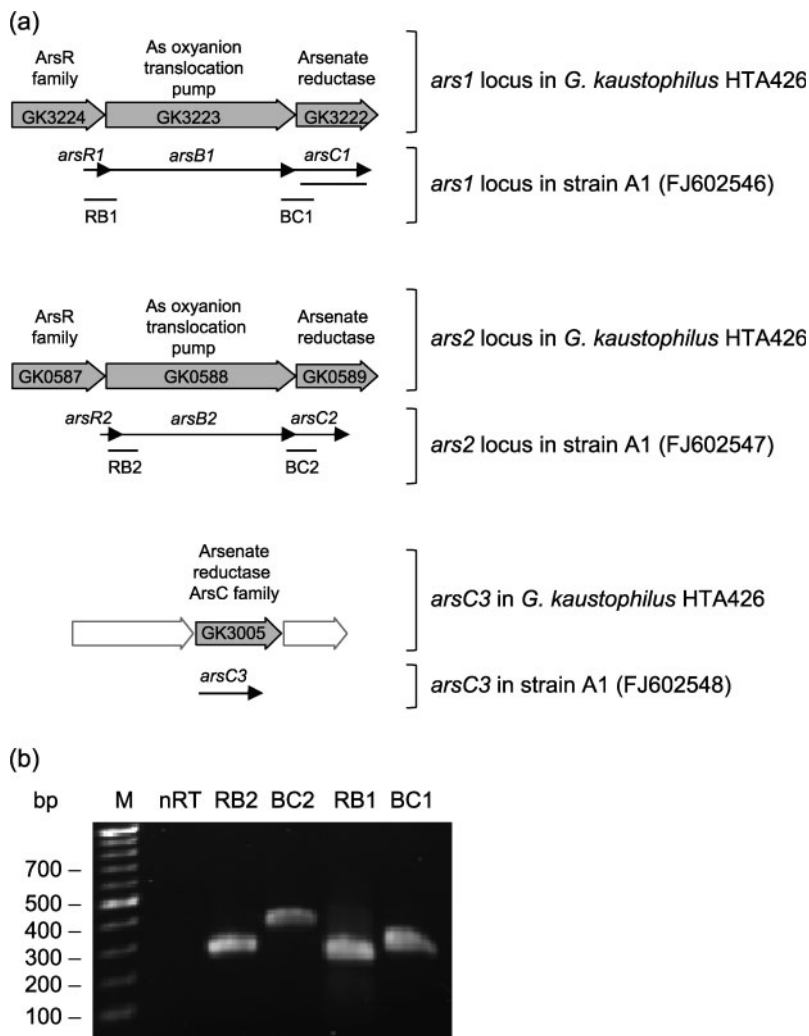


Fig. 2. Organization of arsenic-resistance genes in *Geobacillus* strains. (a) Three arsenic-resistance loci in *G. kaustophilus* HTA426 (redrawn from the genome annotation project) and the corresponding organization of the homologous operons derived for *G. kaustophilus* A1, projected from sequence data. Open arrows represent genes not involved in arsenic resistance. Segments correspond to sequenced regions that were assembled and deposited in GenBank, as indicated by the accession numbers in parentheses. (b) Co-transcription analysis relative to the *ars1* and *ars2* operons. M, DNA markers; nRT, control without reverse transcriptase; RB2, BC2, RB1 and BC1, RT-PCR products corresponding to the regions indicated in (a).

reductases belonging to this family require thioredoxin for arsenate reduction. The ArsC2 of GK is 85 % identical to the arsenate reductase of *Bacillus subtilis* strain 168, an enzyme for which the structure has been solved and has been key to understanding the mechanisms of thioredoxin–arsenate reduction (Li *et al.*, 2007; Bennett *et al.*, 2001). In addition, the protein sequences of the two As(V) reductases ArsC1 and ArsC2 show a high level of similarity to their homologues in the genomes of other *Geobacillus* species, including *Geobacillus thermodenitrificans* NG80-2, *Geobacillus thermoglucosidasius* C56-YS93, *Geobacillus thermoleovorans*, and *Geobacillus* sp. C56-T3, Y412MC52 and Y412MC10.

ORF GK3005, and the partial homologue sequence of strain A1, encode an arsenate reductase (ArsC3, 121 aa) which belongs to a second family of arsenate reductases, ArsC (Fig. 3). Sequences belonging to the ArsC family, first described in *E. coli* (Kaur & Rosen, 1992; Silver *et al.*, 1981), rely on reducing equivalents from glutathione (GSH) and are characterized by a thioredoxin (TRX) fold domain, a motif also found in thioredoxins and glutaredoxins. Because this family of reductases requires glutaredoxin to reduce

arsenate, we used the GK genome as a guide and examined the region surrounding the *ars* operons and *arsC3* in the GK annotation. Two genes encoding putative glutaredoxins were present in the genome, but in locations distant from the *ars3* locus. Instead, a thioredoxin gene was identified approximately 1.3 kb downstream of *arsC3*, although its presence might be unrelated to the function of ArsC3.

Co-transcription of *arsR*, *-B* and *-C* in *ars1* and *ars2*

To demonstrate that *ars1* and *ars2* constitute functional operons, their transcriptional analysis was carried out by RT-PCR. Primers for the detection of specific *arsC* transcripts, and for co-transcription analyses, were designed on the basis of the nucleotide sequences determined for *Geobacillus* strain A1 (Supplementary Table S1). RT-PCR analysis of the mRNA transcripts, using primers that amplified across contiguous genes, demonstrated that three genes, *arsRBC*, were co-transcribed in both operons, as indicated by segments RB1, BC1, RB2 and BC2 (Fig. 2b). The negative control nRT did not include the reverse transcriptase.

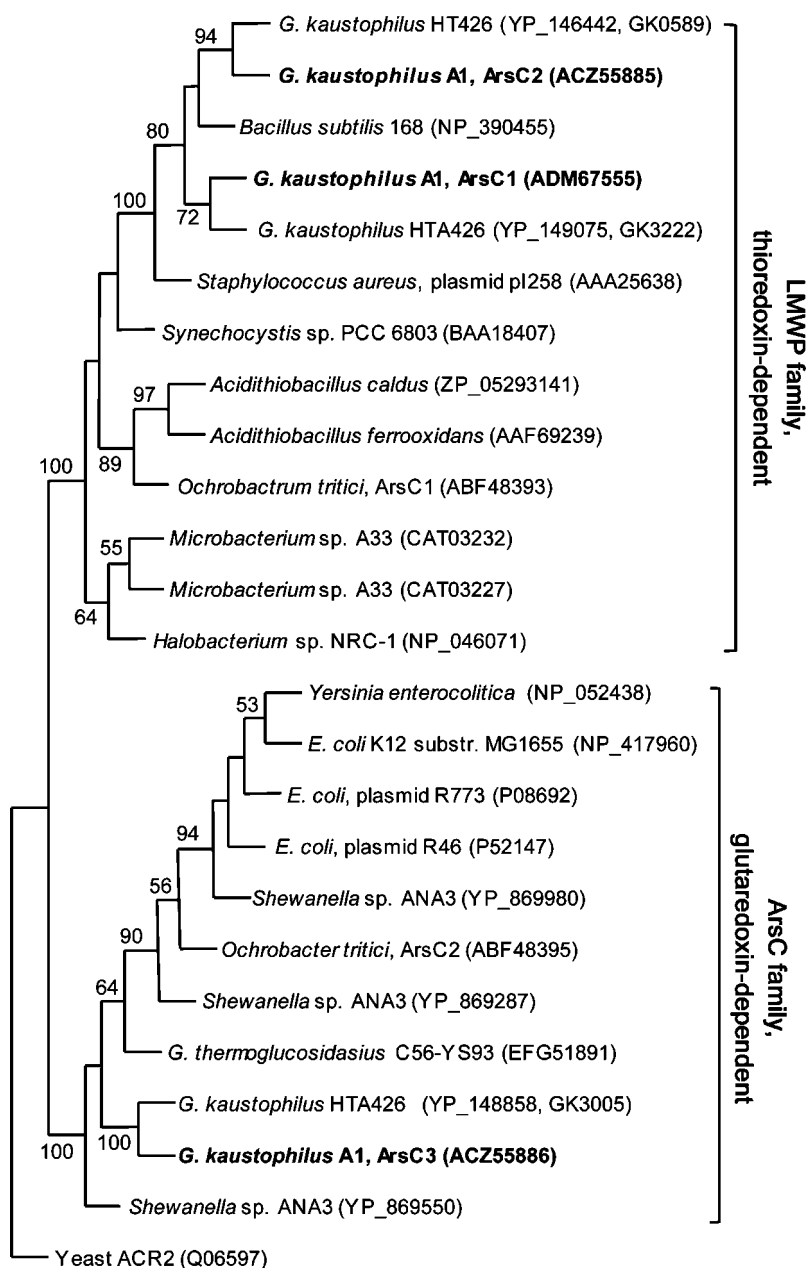


Fig. 3. Maximum-parsimony analysis based on 111 positions of arsenate reductase sequences. Protein accession numbers are in parentheses. Only bootstrap values greater than 50 are shown. The ACR2 yeast sequence was used as the outgroup.

Furthermore, the three reductases were transcribed in both the presence and the absence of arsenate (Fig. 4).

Arsenate reductase genes are induced by arsenate

Since transcripts corresponding to both the *ars1* operon and the *ars2* operon and to *arsC3* were detectable, regardless of the presence of arsenate, to further investigate the role of the three putative *arsC* genes the abundance of their transcripts was measured by qRT-PCR. Cells grown on LB to mid-exponential phase were treated with 5 mM arsenate, and after 10 min total RNA was isolated. The abundance of each transcript, compared with untreated

samples, is shown in Fig. 4. Expression of *arsC1* and *arsC2* was strongly induced in cells grown on arsenate, while the expression of *arsC3* was minimally affected by arsenate. Furthermore, in the absence of arsenate, the abundance of the *arsC3* transcript was slightly more elevated than that of *arsC1* and *arsC2*. These results indicate that both *arsC1* and *arsC2* are responsive to arsenate, presumably through the arsenite resulting from As(V) reduction after cell uptake.

DISCUSSION

A *G. kaustophilus* strain, designated A1 and isolated from a geothermal environment in Tuscany (Italy), was found to be highly resistant to arsenate, and also tolerated moderate

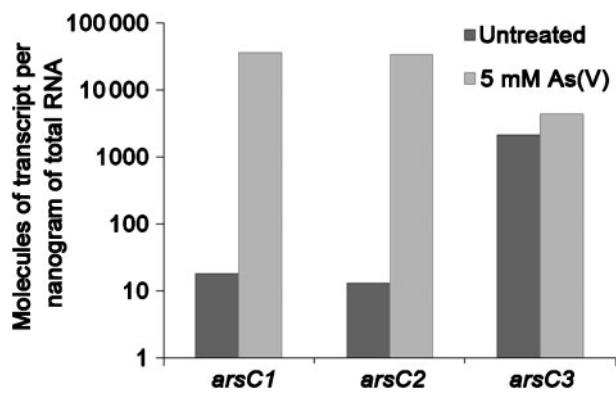


Fig. 4. Expression profile of the arsenate reductase coding genes *arsC1*, *arsC2* and *arsC3* in strain A1. Transcripts were quantified by real-time RT-PCR as molecules of transcript per nanogram of total RNA isolated from cultures grown with 5 mM arsenate (*arsC1*, 36 823 ± 3933; *arsC2*, 33 883 ± 4616; *arsC3*, 4507 ± 142) or without treatment (*arsC1*, 18 ± 7; *arsC2*, 13 ± 1; *arsC3*, 2126 ± 200). Results are reported as means of at least three experiments.

levels of arsenite and antimonite, and formed dark colonies in the presence of tellurate (Cuebas *et al.*, 2011). In this report, we demonstrate that the resistance of strain A1 to arsenic is due to its ability to reduce As(V) to As(III), consistent with the presence of multiple genes encoding putative arsenate reductases and arsenite transporters, although additional mechanisms responsible for arsenic resistance cannot be excluded. The most common systems of arsenate resistance rely on the reduction of arsenate and the active efflux of the resulting arsenite (Rosen, 2002). In prokaryotes, arsenate reduction may be carried out for detoxification or for energy conservation, as an alternative method of anaerobic respiration (Oremland & Stolz, 2003; Stolz *et al.*, 2006). However, fundamentally different enzymes are responsible for these two modes of arsenate reduction (Messens & Silver, 2006; Stolz *et al.*, 2002). No growth was observed when *Geobacillus* strain A1 was incubated in the presence of 5 mM arsenate as the sole terminal electron acceptor (Cuebas *et al.*, 2011). This observation, together with the fact that no Arr sequences were identified in strain A1, indicates that this micro-organism does not respire arsenate. However, reduction of arsenate to arsenite was detected during aerobic growth (Fig. 1), suggesting that the transformation is mediated by a detoxifying arsenate reductase. To verify this hypothesis, specific PCR primers targeting known arsenic detoxification genes were designed on the basis of the chromosome sequence of GK. Such primers enabled the detection of one arsenate reductase of the ArsC family and two unlinked operons, each encoding a typical *arsRBC* cluster, within strain A1.

The sequences of *ars* genes are highly conserved in the two strains A1 and GK, and their arrangement in strain A1 resembles very closely the organization of homologous

genes in GK (Fig. 2). The genome sequences of several *Geobacillus* species are currently available, and from their inspection we observed that most of them (including *G. thermodenitrificans* NG80-2, *G. thermoglucosidasius* C56-YS93, *G. stearothermophilus*, and *Geobacillus* sp. Y412MC10, Y412MC61, C56-T3 and Y4.1MC1) possess a monocistronic reductase characterized by a TRX fold (ArsC family) and at least one *arsRBC* operon encoding an ArsC of the LMWP protein family (Supplementary Fig. S1). In contrast, *Geobacillus* sp. WCH70 encodes one arsenate reductase of each type, but none of them is part of an operon. GK and strain A1 appear unusual within their genus, because these strains possess two copies of the *arsRBC* operon (Fig. 2 and Supplementary Fig. S1). This characteristic is shared with *B. subtilis* (Supplementary Fig. S1), while other *Bacillus* species inspected at the J. Craig Venter Institute Comprehensive Microbial Resource (JCVI-CMR) database encode a single *ars* operon in their chromosome. It should be noted that the *arsC* in one of the two *ars* operons of *B. subtilis* is interrupted by a frameshift. This may explain the difference at the level of arsenate tolerance between *G. kaustophilus* and *B. subtilis* (80 and 4 mM, respectively) (Cuebas *et al.*, 2011; Sato & Kobayashi, 1998) and the loss of arsenic resistance in *Bacillus* mutants lacking the *ars* operon of the skin element (Sato & Kobayashi, 1998). It has been proposed that multiple *ars* operons contribute to elevated arsenate resistance (Li & Krumholz, 2007), although it cannot be excluded that unusual arrangements of genes or their mode of regulation may also contribute to an increased resistance. The most arsenic-resistant species have been shown to rely on typical detoxification genes, chromosomally encoded but organized in unique ways: *Microbacterium* sp. strain A33 encodes an operon containing three arsenate reductases, one of which is fused to an *arsR*-like gene (Achour-Rokbani *et al.*, 2010); *Ochrobactrum tritici* and *Corynebacterium glutamicum* have two structurally different chromosomal operons encoding arsenic-resistance genes (Branco *et al.*, 2008; Ordóñez *et al.*, 2005); and *Desulfovibrio desulfuricans* possesses an *arsRBCC* operon and one *arsC* coding gene located in a separate chromosomal region (Li & Krumholz, 2007). These bacteria display levels of arsenate resistance ranging from 50 mM (*D. desulfuricans*) to 800 mM (*Microbacterium* sp. A33). Except for *Ochrobactrum*, none of the other species mentioned possesses *arsD* or *arsA* sequences. Nevertheless, other factors are likely to contribute to elevated arsenic resistance, as suggested by the observation that '*Ferroplasma acidimanus*', an archaeon resistant to >100 mM arsenate, lacks arsenate reductase genes. Its chromosome encodes only an *arsRB* operon inducible by arsenite (Baker-Austin *et al.*, 2007; Gihring *et al.*, 2003). Other factors of resistance may involve strategies that render the cell membrane less permeable to arsenic compounds, such as downregulation of uptake systems, or yet to be identified genes that enhance the efficiency of the *ars* components.

Using RT-PCR we demonstrated that contiguous ORFs identified as putative arsenic-resistance genes in *G.*

kaustophilus strain A1 were co-transcribed, constituting functional operons. Furthermore, the expression profiles of the three arsenate reductase coding genes, obtained by qRT-PCR, led us to develop a preliminary model for regulation of arsenic resistance in *G. kaustophilus* (Supplementary Fig. S2). Arsenate is easily imported by cells through phosphate transporters (Rosen, 2002). Given that the GK genome encodes the components of the phosphate-specific transport (Pst) system, we speculated that strain A1 could take up As(V) through a similar system. The initial reduction of As(V) is probably carried out by ArsC3, the glutaredoxin-dependent reductase provided by the constitutive expression of *arsC1*. The As(III) that is generated during this reaction binds to both ArsR regulators, which are released from their respective promoters, thereby derepressing the *ars1* and *ars2* operons. The increased expression of the two arsenic-resistance operons results in the accumulation of transporters for As(III) efflux and an additional arsenate reductase, enabling the cells to tolerate high levels of arsenate in their surroundings. A similar model has been proposed for the arsenate response in *D. desulfuricans* (Li & Krumholz, 2007), although the micro-organism has a single *ars* operon, and all of its three arsenate reductases belong to the LMWP family (Li & Krumholz, 2007). The proposed model is to be considered a starting point to describe the elevated arsenic resistance of *G. kaustophilus*. Pending physiological questions include: what is the contribution of each *ars* gene to arsenic resistance, and what other factors, if any, might be responsible for the survival of the microbe in the presence of high arsenic levels? Furthermore, the fine details of the regulation of the *ars* genes remain the subject of further studies.

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