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Chromosomally encoded arsenical resistance of the moderately thermophilic acidophile *Acidithiobacillus caldus*

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Abstract Arsenical resistance is important to bioleaching microorganisms because these organisms release arsenic from minerals such as arsenopyrite during bioleaching. The acidophile *Acidithiobacillus caldus* KU was found to be resistant to the arsenical ions arsenate, arsenite, and antimony via an inducible, chromosomally encoded resistance mechanism. Because no apparent alteration of the toxic ions was observed, *Acidithiobacillus (At.) caldus* was tested to determine if it was resistant as a result of decreased accumulation of toxic ions. Reduced accumulation of arsenate and arsenite by induced *At. caldus* cells supported this hypothesis. It was also found that, with the addition of an energy source, induced *At. caldus* could transport arsenate and arsenite out of the cell against a concentration gradient. The lack of efflux in the absence of an added energy source and in the presence of inhibitors suggested that efflux was energy dependent. Induced *At. caldus* also expressed arsenate reductase activity, indicating that *At. caldus* has an arsenical resistance mechanism that is analogous to previously described systems from other Bacteria. Southern hybridization analysis showed that *At. caldus* and other gram-negative acidophiles carry an *Escherichia coli arsB* homologue on the chromosome.

Key words Arsenic · Arsenite · Arsenate · Antimony · Resistance · Acidophile · Moderate thermophile · *Acidithiobacillus caldus*

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Introduction

The moderately thermophilic, gram-negative acidophile *Acidithiobacillus (At.) caldus* (formerly *Thiobacillus caldus*) (Hallberg and Lindström 1994) is present, as part of a consortium of microorganisms, in industrial bioreactors processing arsenic-containing minerals for the enhanced recovery of gold (Rawlings 1998). Recent work by us has shown that the oxidation of arsenopyrite by the moderately thermophilic *Sulfobacillus thermosulfidooxidans* is enhanced by the presence of *At. caldus* (Dopson and Lindström 1999). A mixed culture containing these two microorganisms exhibited a higher leaching rate, relative to an arsenic-sensitive *Sulfolobus* culture, with an arsenic-containing mineral concentrate (Hallberg et al. 1996c). This information indicates that the acidophile *At. caldus* is arsenic resistant and that such a phenotype is highly desirable from a commercial point of view.

Even though acidophilic bacteria live in environments containing a high concentration of soluble metals, little research has been focused on the metal resistance mechanisms of these bacteria. The effects of several metals, including arsenic, on the growth of *Acidithiobacillus (At.) ferrooxidans* (Tuovinen et al. 1971) and that of arsenite on *At. caldus* (Hallberg et al. 1996a) have been studied. Additionally, *At. ferrooxidans* and *At. thiooxidans* strains were isolated from a continuous laboratory-scale arsenopyrite biooxidation reactor and their arsenic tolerance assessed (Collinet and Morin 1990). However, these experiments did not directly investigate resistance mechanisms toward arsenic; only recently has the arsenic resistance of *At. ferrooxidans* been addressed (Butcher et al. 2000). In that study, the genes encoding arsenic resistance, *arsCRBH*, were cloned and shown to be chromosomally located. The *At. ferrooxidans ars* genes were homologous to the well-

studied genes from neutrophilic bacteria (Rensing et al. 1999).

We report here studies on the arsenic resistance mechanism of *At. caldus*. We show that *At. caldus* is able to resist arsenical ions by an inducible efflux of the ions arsenite, arsenate, and antimony, a mechanism analogous to that in other arsenic-resistant microbes. We also show that *At. caldus* and other gram-negative acidophiles carry an *Escherichia coli arsB* homologue on their chromosomal DNA, implying that arsenical resistance is probably a highly conserved trait among these microorganisms.

Materials and methods

Chemical reagents and analysis

All stock solutions were prepared from commercially available chemicals of the highest quality obtainable. When indicated, the toxic arsenical ions were added as the following salts from stock solutions made in the basal salts solution used to prepare the growth media for acidophiles: arsenite, $NaAsO_2$; arsenate, $Na_2HAsO_4{\cdot}7H_2O$; and antimony, $K(SbO)C_4H_4O_6{\cdot}0.5H_2O$. Radiolabeled arsenate ($Na_3{}^{73}AsO_4$) was purchased from Los Alamos National Laboratory (Los Alamos, NM, USA). Radiolabeled arsenite was prepared by chemical reduction of the arsenate (Reay and Asher 1977). Arsenite and arsenate in the culture supernatants were assayed by high pressure ion chromatography (Sehlin and Lindström 1992).

Bacterial strains and plasmids and growth conditions

The bacteria, plasmids, and bacteriophage used in this study are shown in Table 1. *Acidithiobacillus* strains were grown at 30°C for the mesophiles and 45°C for the moderate thermophiles in growth medium with CO_2-enriched air (2% v/v) (Hallberg et al. 1996b). When needed, media were solidified with 1.5% (w/v) bacteriological agar no. 1 (Oxoid, Basingstoke, UK), essentially as previously described (Lindström and Sehlin 1989), with the exception that the pH of the double-strength mineral salts solution was adjusted to 1.90 to ensure a final pH of 2.5 in the solid media. Potassium tetrathionate (5 mM) served as the energy source. Other acidophilic microorganisms were grown in media given in the reference for each microbe (see Table 1). *E. coli* was grown at 37°C in Luria-Bertani (LB) medium according to standard techniques (Sambrook et al. 1989).

Isolation of constitutively resistant mutants

Constitutively arsenic-resistant mutants were enriched by growing *At. caldus* in the presence of 10 mM arsenite until early exponential growth phase, an optical density at 440 nm (OD_{440nm}) of 0.260–0.270, as measured on a Hitachi (Tokyo,

Table 1. Bacterial strains, phage, and plasmids used in this study

Bacteria, phage, plasmids	Genotype and phenotype	Reference or source
Escherichia coli		
AW3110	K12 F^- IN(*rrnD-rrnE*) Δ*ars*::*cam*	Carlin et al. (1995)
MC1061	*HsdR mcrB araD*139 Δ(*ara*ABC-*leu*)7679 Δ*lac*X74 *galU galK rpsL thi*	Meissner et al. (1987)
MC1061Δ*ars*::*cam*	P1 transduction of Δ*ars*::*cam* from AW3110	This study
Acidithiobacillus (At.) caldus		
KU	Wild type, moderate thermophile, type strain	ATCC 51756
KU308	Arsenical resistance constitutively expressed, moderate thermophile	This study
At. ferrooxidans		
ATCC 23270	Wild type, mesophile, type strain	ATCC
ATCC 19859	Wild type, mesophile	ATCC
At. thiooxidans		
ATCC 19377	Wild type, mesophile, type strain	ATCC
T1D-1	Wild type, recent isolate	This laboratory
Thiomonas cuprina		
DSM5495	Wild type, mesophile, type strain	Huber and Stetter (1990); Moreira and Amils (1997)
Acidiphilium acidophilum		
ATCC 27807	Wild type, mesophile, type strain	ATCC
Acidocella facilis		
ATCC 35904	Wild type, mesophile, type strain	ATCC
Phage		
P1*vir*		Lab stock
Plasmids		
pUM3	4.3-kb fragment of R773 cloned into pBR322	Mobley et al. (1983)
pKE462	7.5-kb fragment of R46 cloned into R300B	Mobley et al. (1984)
pMC1871	Fusion vector for production of hybrid proteins with *E. coli lacZ* gene	Pharmacia
pMGD1	640-bp PCR product from pKE462 with a functional *arsR* and partial *arsD* cloned into pMC1871	This study

Japan) 150-20 spectrophotometer in a 1-cm cuvette. The bacteria were then harvested by centrifugation and resuspended in medium lacking arsenite. Following growth to stationary phase, this culture was inoculated to 10% (v/v) into fresh medium containing 10 mM arsenite and grown again to $OD_{440nm} = 0.1$.

After three such cycles, the bacteria were plated onto solid medium lacking any of the arsenical ions, and constitutive mutants were identified by replica plating onto solid medium containing 5 mM arsenite. One such mutant (KU308) was confirmed as constitutively resistant by measuring growth in liquid medium containing 10 mM arsenite after growth for several generations in the absence of arsenical ions.

Induction of arsenical resistance

To study the induction of arsenical resistance, *At. caldus* strains were grown overnight in medium containing either 50 μM antimony, 100 μM arsenite, or 1 mM arsenate. Uninduced bacteria were grown in the absence of any of these toxic ions. These cultures served as inocula for fresh media containing no arsenical ions, as a control, or into media containing either 5 mM arsenite, 100 mM arsenate, or 100 μM antimony. Growth was then determined by measuring OD_{440nm} after 8 h.

Arsenic transport assays

Resting cells of *At. caldus* were prepared by harvesting strains KU or KU308 during late exponential growth phase by centrifugation (10 min at 9,000*g*), washing, and resuspending in β-alanine buffer (50 mM β-alanine adjusted to pH 3 with H_2SO_4). The resulting resting cells were kept on ice and used within 2 h.

For arsenic uptake studies, the bacteria were resuspended to a final concentration of 1 mg ml^{-1} (dry wt) in β-alanine buffer (1 ml total volume); the mixture was shaken at 150 rpm and 45°C for 5 min in the presence of inhibitors as indicated. Each experiment was initiated by the addition of thiosulfate to a final concentration of 2 mM and radioactive arsenate or arsenite (4 μCi ^{73}As ml^{-1}) and 50 nmol cold arsenate or arsenite. Samples of 100 μl were withdrawn and filtered (0.22-μm-pore-size cellulose acetate Sartorius filter) at the indicated times. After washing with 2 ml β-alanine buffer, the filters were placed into scintillation vials, covered with 3 ml scintillation cocktail (Wallac Optiphase "HiSafe"; Wallac, Turku, Finland), and counted with a Wallac 1409 liquid scintillation counter. To ascertain how much of the radioactive arsenic had been taken up, 100 μl of the reaction mixture was added directly to a scintillation vial and counted as before; this step gave the total counts per minute (cpm) in 100 μl of the reaction mixture.

Arsenic efflux was assayed by harvesting *At. caldus* KU and KU308 at late exponential growth phase, then washing the cells once with 100 ml 100 mM H_2SO_4 and twice with 1 ml 100 mM H_2SO_4. The resulting pellet was resuspended in 100 μl 100 mM H_2SO_4/100 ml of the start culture containing a total of 1 μCi arsenate or arsenite and 50 μg chloramphenicol ml^{-1} to prevent induction to arsenical resistance. This mixture was incubated at 45°C for 30 min; the loaded cells were then washed three times in β-alanine buffer. The cells were diluted to a final concentration of 1 mg ml^{-1} in β-alanine (total volume, 1 ml) and preincubated at 45°C for 5 min in the presence of 50 nmol cold arsenate or arsenite and inhibitors as indicated. The reaction was started by the addition of thiosulfate to 2 mM (unless otherwise indicated), and at the specified times 100-μl samples were filtered, washed, and counted as for the uptake experiments. All uptake and efflux experiments were carried out in triplicate, and averages and standard deviations are presented ($n = 3$, except where stated).

Arsenate reduction assay

Initial attempts to study arsenate reduction by *At. caldus* using a thin-layer chromatography- (TLC-) based assay (Ji and Silver 1992) were unsuccessful. We therefore sought to study reduction using a nonradioactive-based assay: we fused a β-galactosidase gene downstream of the *arsR* gene to the 24th nucleotide of *arsD* to make a sensitive arsenite reporter. The production of arsenite by *At. caldus* was measured as an increase in β-galactosidase activity in the *E. coli* reporter. The gene fusion was created by amplifying a 640-bp fragment of pKE462 containing the complete *arsR* gene and a part of the *arsD* gene by polymerase chain reaction (PCR) using the primers ArsRF (5′-TTTTCCCGGGTCGTTTTTCAACCGCA-3′) and ArsRR (5′-TTTTCCCGGGTTTCAGCCATTGCAC-3′). The amplified fragment was cloned into the unique *Sma*I site in pMC1871, yielding the plasmid pMGD1.

Escherichia coli MC1061Δ*ars*::*cam* was created by making a P1 library of AW3110 and transducing (Miller 1992) the gene library into MC1061. Transductants were identified by plating on LB plates containing 20 μg chloramphenicol ml^{-1}, and the resulting colonies were tested for the absence of *ars* genes by colony hybridization (Sambrook et al. 1989) with a fragment of *arsB* gene as probe (see following). The *E. coli* arsenite reporter strain was generated by transforming MC1061Δ*ars*::*cam* with pMGD1, followed by selection on LB plates containing chloramphenicol and tetracycline (15 μg ml^{-1}).

Arsenate reduction with uninduced and induced (by growth in the presence of 100 μM arsenite) cultures of *At. caldus* KU was carried out with 1 mg ml^{-1} (dry wt) of cells in sulfate buffer (50 mM Na_2SO_4 and 50 mM K_2SO_4, adjusted to pH 3 with H_2SO_4). The cell suspensions were preincubated at 45°C for 5 min, after which 100 mM arsenate and tetrathionate (to 1 mM) were added. Samples (100 μl) were removed and filtered (0.22-μm cellulose acetate filter), and these filtrates were frozen in liquid N_2. Any arsenite produced from the reduction of arsenate by *At. caldus* was detected by measuring β-galactosidase activity (Miller 1992) in the *E. coli* reporter strain 2 h after the filtrates from the various *At. caldus* cell suspensions, diluted 1,000-fold, were added. Controls included incubating induced KU in the absence of arsenate or in the absence of an energy source. Additionally, the chemical reduction of arsenate was tested by adding 5 mM thiosulfate or 5 mM sulfite to

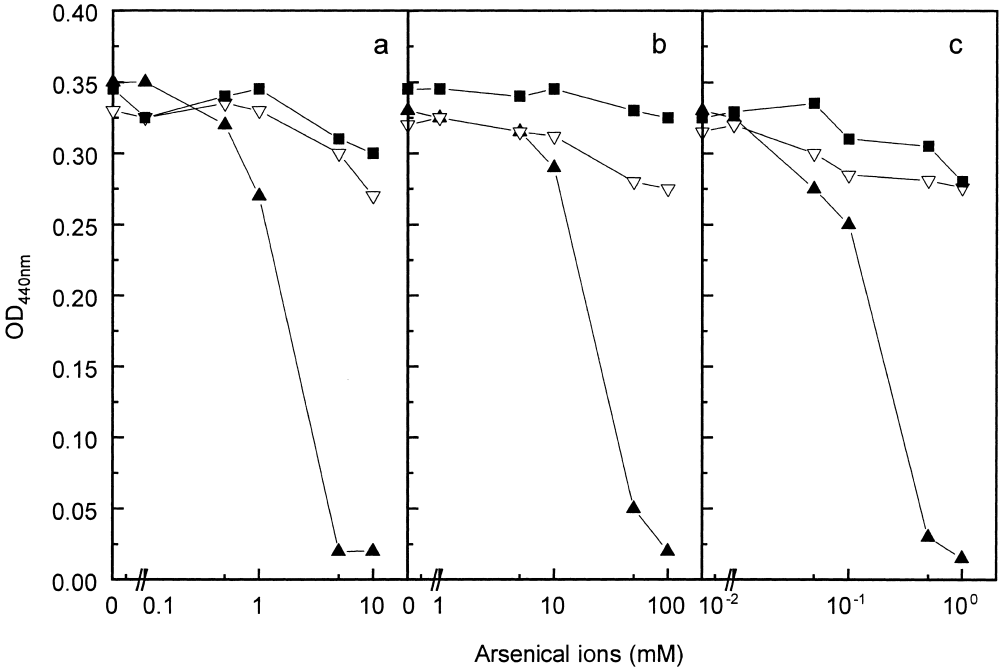

Fig. 1a–c. Resistance of *Acidithiobacillus (At.) caldus* to arsenical ions shown by final optical density at 440 nm (OD_{440nm}) of *At. caldus* strains after 8 h growth in the presence of arsenite (**a**), arsenate (**b**), and antimony (**c**). Strain KU was grown in the absence (*solid triangles*) or presence (*open inverted triangles*) of 100 µM arsenite, and strain KU308 was grown in the absence of arsenic (*solid squares*), before inoculation into arsenical-containing medium

arsenate-containing buffer. For each sample of an *At. caldus* cell suspension, β-galactosidase activity was determined in triplicate; therefore, an *n* value of 9 corresponds to three separate reduction assays.

Southern hybridization analysis

Chromosomal DNA was prepared from acidophilic bacteria using the minipreparation method developed for gram-negative bacteria (Wilson 1987). The DNA from each strain was digested with *Pst*I before agarose gel electrophoresis and blotting the DNA onto nylon membranes (Hybond+; Amersham, Uppsala, Sweden). Hybridization was carried out according to the manufacturer's instructions, using a 364-bp fragment of the *arsB* gene as probe. The probe was prepared by PCR amplification using the primers ArsBF (5′-TTTATCGCCGTTATTATCAT-3′) and ArsBR (5′-CAAGTTCGATACGATAAGCG-3′) and plasmid pUM3 (Mobley et al. 1983) as template. The probe was labeled with [α^{32}P]dATP using the Amersham RPN1604 Megaprime DNA labeling system according to the protocol provided. Following hybridization, the membrane was washed twice for 15 min under moderate stringency, 0.5× SSC (1× SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0) at 45°C, before overnight exposure to X-ray film.

Results

Induction of arsenical resistance

We have previously shown that arsenite is bactericidal to *At. caldus* (Hallberg et al. 1996a). On prolonged incubation of cultures poisoned by arsenite, growth of *At. caldus* occurred, indicating that resistant cells accumulated. To determine if this resistant population was induced to arsenic resistance, these cells were grown in the absence of arsenite for three generations and then inoculated into arsenite-containing medium. Once again, the arsenite proved toxic, indicating that *At. caldus* cells that were resistant to arsenite did not accumulate (data not shown).

Cells of *At. caldus* KU were unable to grow during an 8-h period in the presence of 5 mM arsenite, 100 mM arsenate, or 100 µM antimony if pregrown in the absence of arsenical ions (Fig. 1). In contrast, if cells of strain KU were grown overnight in the presence of a nontoxic concentration of arsenite (100 µM), they grew to a high optical density after 8 h in the presence of 5 mM arsenite, 100 mM arsenate, or 100 µM antimony (Fig. 1). Growth of *At. caldus* KU in the presence of 1 mM arsenate or 50 µM antimony produced the same effect as growth in the presence of arsenite (data not shown), which indicated that resistance to each of the toxic arsenical ions is inducible by the other arsenical ions. The bacteria had to be growing to be induced to arsenical resistance because arsenite added to stationary-phase cultures did not induce arsenite resistance.

Isolation of constitutively resistant mutants

Because the arsenical resistance phenotype was inducible in *At. caldus*, we attempted to isolate constitutively resistant mutants. After three rounds of enrichment, approximately 10% of the total colonies grew when replicated from arsenite-free plates onto solid medium containing 5 mM arsenite. These colonies were taken from the tetrathionate master plate and the constitutively resistant phenotype was confirmed by growth in liquid medium containing 5 mM arsenite without prior induction of the isolates. One isolate thus obtained, *At. caldus* KU308, was able to grow in medium

containing 5 mM arsenite, 100 mM arsenate, and 100 μM antimony even though it had not been pregrown in the presence of any arsenical ion (see Fig. 1).

Toxicity of arsenical ions in spent culture medium

The concentration of neither arsenite nor arsenate decreased in the culture medium of induced *At. caldus* strain KU as determined by ion chromatography. We also tested spent culture medium, containing 10 mM arsenite, from an induced culture of *At. caldus* KU for toxicity. Induced bacteria could grow in filter-sterilized arsenite-containing spent culture medium whereas uninduced bacteria were unable to grow (data not shown). Uninduced *At. caldus* KU could grow in spent culture medium lacking arsenite. It thus appeared that *At. caldus* KU did not alter arsenic to a nonionic form or remove arsenic as a means of resistance (antimony detoxification was not tested).

Arsenic transport assays

Because there was no apparent detoxification of the arsenic, we investigated transport of the toxic ions by the bacteria. The wild-type strain *At. caldus* KU was used as the uninduced strain and the constitutively resistant mutant KU308 was used as the induced strain to avoid any carryover of arsenic that might influence the transport assays. Uninduced KU took up arsenate in the presence of thiosulfate but did not take up the arsenate if no energy source was supplied (Fig. 2a). The constitutively resistant mutant KU308 grown in the absence of arsenical ions took up little arsenate in the presence of thiosulfate (Fig. 2a). Arsenite accumulation in KU did not require the presence of an energy source (Fig. 2b). Strain KU308 exhibited an intermediate level of arsenite uptake in the absence of an energy source and even less uptake with thiosulfate (Fig. 2b).

The amount of arsenite inside KU308 rose over the time course of the experiment but never reached the level observed in KU. The presence of any of the inhibitors tested resulted in little uptake of arsenate by either KU or KU308 (Table 2), whereas they caused an increased accumulation of arsenite in both KU and KU308 (Table 2). These results indicated that resistance to arsenical ions was due to energy-dependent reduced accumulation of the toxic ions by the bacteria.

This reduced uptake of arsenate by arsenic-induced *At. caldus* strains was presumed to be a result of rapid efflux of the accumulated arsenate or arsenite. Conditions were established to allow the uptake of arsenate or arsenite by *At. caldus* in the absence of an energy source, and the amount of 73-arsenic remaining in cells that had been reenergized was measured. *At. caldus* KU treated in such a manner transported 16 ± 7% ($n = 3$) of the accumulated arsenate during a 10-min period with 2 mM thiosulfate as the energy source (Table 2). Under the same conditions, KU308 expelled 51 ± 5% ($n = 3$) of the accumulated arsenate against a concentration gradient (Table 2). No further increase in efflux of arsenate occurred with the addition of thiosulfate to 8 mM (data not shown).

Acidithiobacillus caldus KU loaded with 73-arsenite did not expel any arsenite in the absence of an energy source and only 10 ± 5% ($n = 3$) of the arsenite in the presence of 2 mM thiosulfate (Table 2). In contrast, KU308 expelled as much as 63 ± 7% of the loaded arsenite against a concentration gradient in the presence of thiosulfate (Fig. 3). The

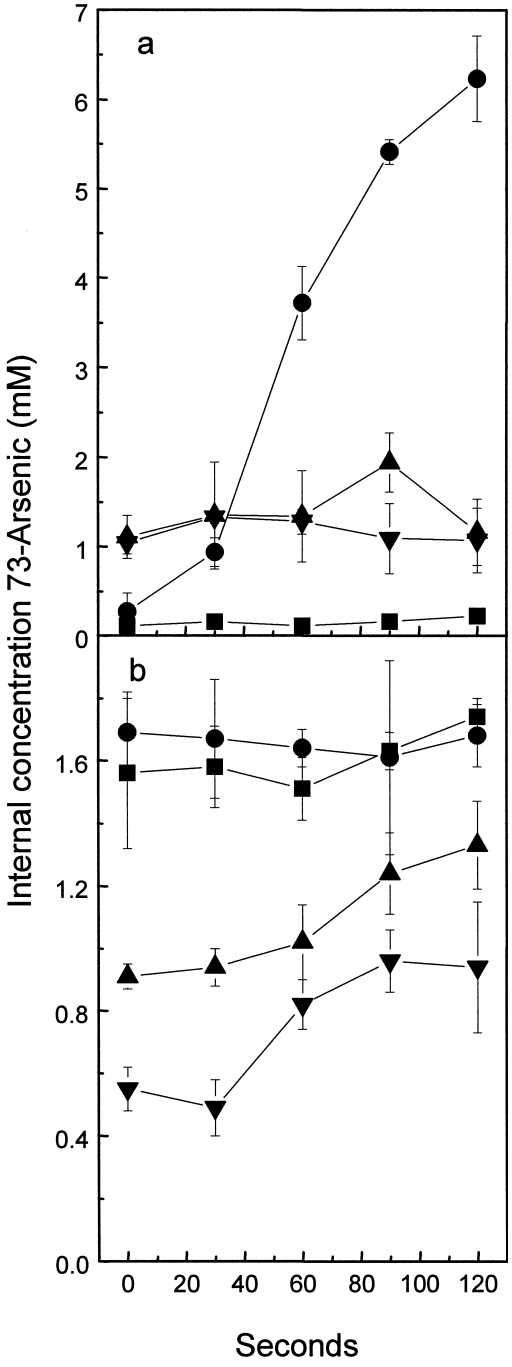

Fig. 2. Uptake of arsenate (**a**) and arsenite (**b**) by *At. caldus*. Strain KU was incubated without thiosulfate (*squares*) or in the presence of 2 mM thiosulfate (*circles*), and strain KU308 was incubated without thiosulfate (*triangles*) or with thiosulfate (*inverted triangles*). Data points represent mean values of three replicates ± SD

Table 2. Uptake and efflux of 73-arsenate and 73-arsenite after 2 and 10 min, respectively, with 2 mM thiosulfate in the absence or presence of inhibitors

Inhibitor	73-Arsenic	*At. caldus* KU		*At. caldus* KU308	
		Uptake (mM)	Efflux (%)[a]	Uptake (mM)	Efflux (%)[a]
None	Arsenate	6.2 ± 0.5	16 ± 7	1.1 ± 0.4	51 ± 13
	Arsenite	1.7 ± 0.1	10 ± 5	0.9 ± 0.2	51 ± 5
20 μM CCCP	Arsenate	0.4 ± 0.1	1 ± 1	1.0 ± 0.1	17 ± 1
	Arsenite	3.0 ± 0.2	6 ± 1	2.9 ± 0.2	12 ± 3
1 mM DCCD	Arsenate	0.9 ± 0.1	11 ± 1	0.9 ± 0.1	3 ± 1
	Arsenite	2.3 ± 0.2	10 ± 1	1.8 ± 0.3	15 ± 1
1 mM azide	Arsenate	0.6 ± 0.1	2 ± 1	0.1 ± 0.03	0 ± 1
	Arsenite	3.5 ± 0.4	8 ± 1	2.4 ± 0.2	11 ± 1

[a]Efflux data are given as percent of arsenic exported from the cells compared to the respective time zero value. Values are averages ± SD (n = 3)
CCCP, 3-chlorcarbonyl cyanide phenylhydrazon; DCCD, N,N′-dicyclohexylcarbodiimide

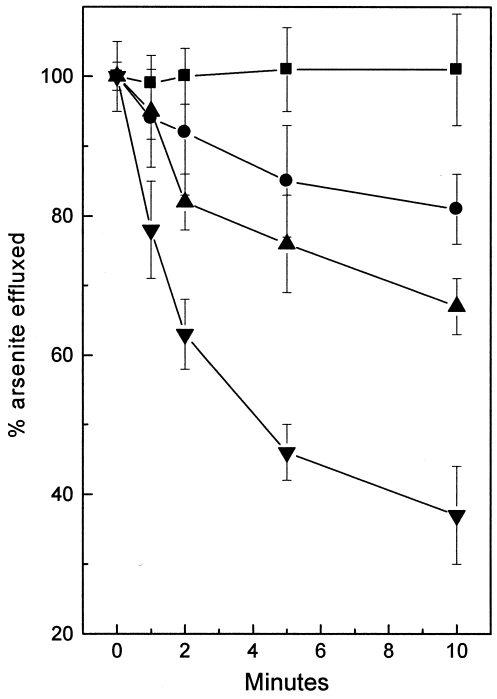

Fig. 3. Efflux of 73-arsenite by KU308. The bacteria were incubated in the absence (*squares*) or in the presence of 10 μM (*circles*), 100 μM (*triangles*), and 8 mM (*inverted triangles*) thiosulfate. *Data points* represent mean values from three experiments ± SD

addition of inhibitors had little effect on the efflux of arsenite and arsenate by KU, but they strongly inhibited efflux from the cell by KU308 (see Table 2).

Reduction of arsenate to arsenite

Acidithiobacillus caldus was tested to determine if it could reduce arsenate to arsenite using *E. coli* MC1061Δ*ars*::*cam* pMGD1 as an arsenite detection system. Filtrates from *At. caldus*-free controls without an energy source containing 100 mM arsenate or arsenite yielded 3,849 ± 1,179 (n = 9) and 9,504 ± 588 (n = 9) Miller units of β-galactosidase activity, respectively, in the *E. coli* reporter strain (Fig. 4). Cell-free controls containing 100 mM arsenate plus thiosulfate or sulfite, potential intermediates of tetrathionate metabolism, gave Miller unit values of 3,935 ± 784 (n = 9) and 4,633 ± 486 (n = 9), respectively. Production of β-galactosidase caused by arsenic-free filtrates of *At. caldus* suspensions was 1,225 ± 189 (n = 12).

Filtrates from uninduced *At. caldus* KU incubated in the presence of 100 mM arsenate resulted in a higher β-galactosidase activity (5,981 ± 865 Miller units) in the *E. coli* reporter strain compared to filtrates from the cell-free control. The addition of tetrathionate as an energy source had little effect (Fig. 4). Filtrates of induced KU without tetrathionate taken at time zero gave slightly higher β-galactosidase activity, 7,363 ± 991 Miller units, and 8,080 ± 1,213 (n = 9) with filtrates taken after 30 s incubation. Filtrates from induced KU+1 mM tetrathionate taken at t=0 s caused an increase in β-galactosidase activity from 7,050 ± 504 Miller units in the *E. coli* reporter strain to 9,430 ± 2,031 Miller units at t = 30 s (Fig. 4); this value then decreased to 7,931 ± 735 (n = 9) Miller units at t = 120 s.

Southern hybridization analysis

All these data implied that *At. caldus* had an arsenic resistance mechanism similar to that of neutrophilic microorganisms. We sought to further investigate this finding using Southern hybridization analysis. Restriction digestion of *At. caldus* chromosomal DNA using several different enzymes resulted in a single DNA fragment that hybridized with the *ars* genes from *E. coli* in each case (data not shown). Further experiments showed that, of the *ars* genes, only the 3′-end of the *arsB* gene exhibited any homology with KU DNA (Fig. 5). Under the same wash conditions and using the same blots that had been stripped of *arsB* probe, no signal was obtained using labeled *arsA* or *arsC* genes as probe. Also, Western blotting using anti-ArsA antibodies did not show any cross-reacting proteins in extracts from induced KU or from KU308 (data not shown).

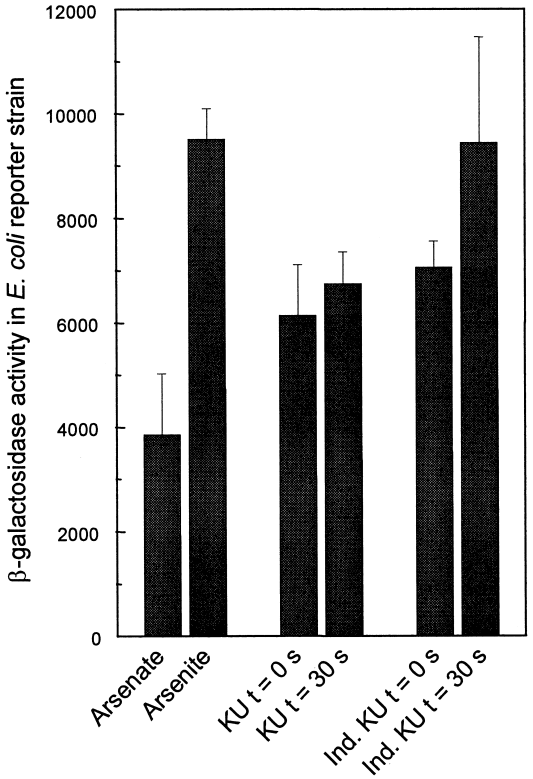

Fig. 4. Reduction of arsenate by *At. caldus* determined as β-galactosidase activity in the *Escherichia coli* strain with *arsRD::lacZ* gene fusion. Activity was measured after adding samples, taken at 0 and 30 s, of cell suspensions of *At. caldus* KU or induced KU that were incubated with tetrathionate and 100 mM arsenate. For reference, β-galactosidase activity was also determined after adding filtrates of sulfate buffer containing 100 mM arsenate or arsenite. Data are average values from three separate experiments ± SD

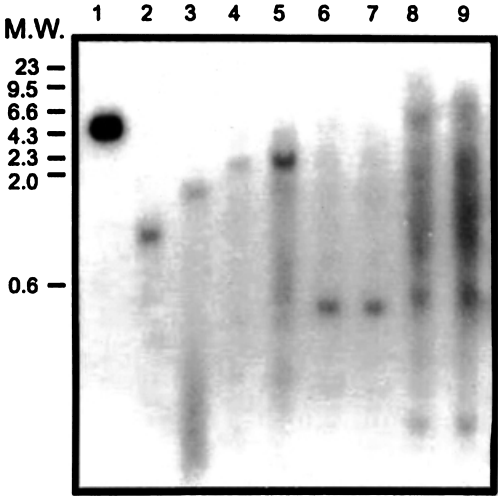

Fig. 5. Southern hybridization of DNA from gram-negative acidophiles with a fragment of the *E. coli arsB* gene as probe. The DNA included the *arsB*-containing plasmid pUM3 (*lane 1*), *At. caldus* KU DNA (*lane 2*), *Thiomonas cuprina* (*lane 3*), *At. thiooxidans* ATCC 19377 (*lane 4*), *At. thiooxidans* T1D-1 (*lane 5*), *At. ferrooxidans* ATCC 19859 (*lane 6*), *At. ferrooxidans* ATCC 23270 (*lane 7*), *Acidiphilium acidophilum* ATCC 27807 (*lane 8*), and *Acidocella facilis* ATCC 35904 (*lane 9*)

The same *arsB* probe was used for hybridization with DNA isolated from other acidophilic bacteria, all belonging to the *Proteobacteria*. As with *At. caldus*, each of the other organisms also had chromosomal DNA that exhibited homology to the *arsB* probe (Fig. 5). This result indicated that all the acidophiles carried an *arsB* homologue on the chromosomal DNA.

Discussion

Because microorganisms catalyzing the oxidation of arsenic-containing metal sulfides release arsenic during bioleaching, arsenic resistance in these microorganisms is important, and yet has received surprisingly little attention. Previously published studies on acidophiles have addressed resistance levels toward arsenic and a few other metals (Collinet and Morin 1990; Garcia and da Silva 1991; Hallberg et al. 1996c) and the mode of toxicity of arsenic to acidophiles (Hallberg et al. 1996a). Only recently has the arsenic resistance mechanism of an acidophile been investigated (Butcher et al. 2000). The data presented in this article represent the first biochemical study of the mechanism of arsenic resistance in acidophiles.

It was found that prolonged exposure of *At. caldus* to the toxic arsenical ions arsenite, arsenate, and antimony resulted in increased resistance to these ions and that the resistance was inducible. Possible resistance mechanisms include alteration of the toxic ions by methylation, as has been found for marine algae (Cervantes et al. 1994), or oxidation of arsenite to arsenate as observed with the acidophile *Sulfolobus* strain BC65 (Sehlin and Lindström 1992). No evidence was found for such detoxification of arsenic by *At. caldus*, and therefore we considered that resistance could have resulted from reduced accumulation of arsenic caused by energy-dependent efflux.

The uptake of arsenate by *At. caldus* probably occurred via the phosphate inorganic uptake system, as shown by a decrease in accumulation in the presence of phosphate ions (data not shown). It was also evident that uptake was energy dependent because up to 6.23 mM arsenate, from an initial concentration of 500 nM, accumulated in uninduced *At. caldus* KU when an energy source was added. The lack of arsenate uptake in the presence of poisons (see Table 2) also suggested that energy was required because all the poisons tested have been found to inhibit energy production in *At. caldus* (Dopson 2001).

As seen by its uptake in the absence of an energy source, arsenite accumulated in *At. caldus* in an energy-independent manner. The level of arsenite uptake by the constitutively resistant *At. caldus* KU308 was initially much lower than by uninduced KU and began to increase after 30 s. This effect is probably caused by energy source depletion, which effectively limited further efflux of the arsenite from the bacteria. The final level of accumulation of arsen-

ite by this strain was only 50% of that shown by the uninduced strain (see Fig. 2).

The reduced accumulation of arsenic by induced cells of *At. caldus* KU or the mutant strain KU308 was the result of active efflux of 73-arsenic. A higher efflux was observed for arsenite (63%) compared to arsenate (51%), probably because of the additional energy demand of arsenate reduction required for the efflux of arsenate. The increased level of efflux observed with the addition of up to 2 and 8mM thiosulfate for arsenate and arsenite (Fig. 3), respectively, compared to cells lacking an energy source, indicates that efflux is energy dependent.

The inhibition of efflux by the poisons tested also suggests that efflux by induced *At. caldus* is energy dependent. Unfortunately, all these poisons inhibited ATP synthesis by *At. caldus* (Dopson 2001), and we were therefore unable to determine if arsenite efflux was driven by ATP (Rosen et al. 1988) or by the membrane potential (Bröer et al. 1993; Cai et al. 1998; Sato and Kobayashi 1998). That a maximum of 63% of the total arsenite was expelled even with the addition of higher concentrations of the energy source may be explained by binding of the arsenite to cellular proteins.

We also tested whether *At. caldus* reduced arsenate using an *arsRD*::*lacZ* gene fusion as an arsenite reporter. Chemical reduction of arsenate caused by the presence of reduced inorganic sulfur compounds resulted in a small increase in Miller units shown by the *E. coli* arsenite reporter, but the increase was insufficient to account for the increase observed in the presence of *At. caldus* or induced *At. caldus*. The increased β-galactosidase activity from the reporter gene in *E. coli* caused by filtered supernatants of induced *At. caldus* KU incubated in the presence of arsenate suggested that arsenate was converted to arsenite.

Filtrates taken after incubation of induced *At. caldus* with an energy source for 30s yielded β-galactosidase activity in the *E. coli* reporter that corresponded to reduction of 99% of the available arsenate to arsenite, decreasing to a value corresponding to conversion of 83% of the available arsenate to arsenite after 2min. This decrease could have been caused by binding of arsenite to cellular proteins that are then removed by filtration before adding cell-free suspensions to the *E. coli* arsenite reporter strain; this result also suggests that some arsenite binds to cellular protein, as was observed during efflux. The slightly higher β-galactosidase activity generated by filtrates of uninduced KU plus arsenate compared to cell-free filtrates with arsenate suggested that uninduced KU had a low level of arsenate reductase activity. This finding is also supported by the low level of efflux observed by uninduced *At. caldus* KU (see Table 2).

Resistance to arsenic via an efflux system has been reported in many organisms, including *E. coli*, *Staphylococcus aureus*, *Staphylococcus xylosus*, *Saccharomyces cerevisiae*, and *Pseudomonas aeruginosa* (reviewed by Xu et al. 1998; Rensing et al. 1999). All the biochemical data obtained with *At. caldus* indicate a similar mechanism of arsenic resistance in this acidophile. Southern hybridization analysis showed that an *arsB* homologue was present on the chromosome of *At. caldus* strain KU (see Fig. 5) and also on that of *At. caldus* strain BC13 (data not shown). Both strains carry a highly similar plasmid (K.B. Hallberg, unpublished data) that did not hybridize with the *arsB* gene probe, supporting the chromosomal location of the homologue.

Hybridization analysis with chromosomal DNA from other acidophiles, all α-, β-, or γ-*Proteobacteria*, indicated that they too carried a chromosomal *arsB* homologue, extending the range of bacteria that carry a chromosomally encoded arsenical resistance operon. No homologue of the *arsA* gene was detected by hybridization under the same low-stringency conditions, nor was any protein that cross-reacted with anti-ArsA antibodies detected in extracts of induced *At. caldus* KU (data not shown), which is consistent with the chromosomal location of the resistance genes for *At. ferrooxidans* (Butcher et al. 2000) and neutrophiles (see Rensing et al. 1999).

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