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Isolation and Characterization of Arsenate-Reducing Bacteria from Arsenic-Contaminated Sites in New Zealand

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Abstract. Two environmental sites in New Zealand were sampled (e.g., water and sediment) for bacterial isolates that could use either arsenite as an electron donor or arsenate as an electron acceptor under aerobic and anaerobic growth conditions, respectively. These two sites were subjected to widespread arsenic contamination from mine tailings generated from historic gold mining activities or from geothermal effluent. No bacteria were isolated from these sites that could utilize arsenite or arsenate under the respective growth conditions tested, but a number of chemoheterotrophic bacteria were isolated that could grow in the presence of high concentrations of arsenic species. In total, 17 morphologically distinct arsenic-resistant heterotrophic bacteria isolates were enriched from the sediment samples, and analysis of the 16S rRNA gene sequence of these bacteria revealed them to be members of the genera *Exiguobacterium, Aeromonas, Bacillus, Pseudomonas, Escherichia,* and *Acinetobacter*. Two isolates, *Exiguobacterium* sp. WK6 and *Aeromonas* sp. CA1, were of particular interest because they appeared to gain metabolic energy from arsenate under aerobic growth conditions, as demonstrated by an increase in cellular growth yield and growth rate in the presence of arsenate. Both bacteria were capable of reducing arsenate to arsenite via a non-respiratory mechanism. Strain WK6 was positive for *arsB,* but the pathway of arsenate reduction for isolate CA1 was via a hitherto unknown mechanism. These isolates were not gaining an energetic advantage from arsenate or arsenite utilization, but were instead detoxifying arsenate to arsenite. As a subsidiary process to arsenate reduction, the external pH of the growth medium increased (i.e., became more alkaline), allowing these bacteria to grow for extended periods of time.

Although arsenic is generally toxic to life, it has been demonstrated that microorganisms can use arsenic compounds as electron donors, electron acceptors, or possess arsenic detoxification mechanisms [2, 7, 15, 20, 23, 28, 29]. The most common oxidation states of arsenic in the environment are the pentavalent As (V) (arsenate) and the trivalent As (III) (arsenite) forms [10]. Of these two, As (III) is more toxic and has been shown to inhibit various dehydrogenases (e.g., pyruvate, α-ketoglutarate, and dihydrolipolate) [12]. Arsenite (AsO_2^- or AsO_3^{2-}) has the ability to bind to sulfhydryl groups of proteins and dithiols such as glutaredoxin. Arsenate (AsO_4^{3-}) acts as a structural analog of phosphate and inhibits oxidative phosphorylation by producing unstable arsenylated derivatives [3, 4, 11].

Resistance to arsenic species in both Gram-positive and Gram-negative organisms results from energy-dependent efflux of either arsenate or arsenite from the cell mediated via the *ars* operon [7, 14, 15, 22, 24]. In *E. coli,* an ArsA–ArsB complex functions as a primary arsenite pump [7]. In *S. aureus,* ArsB alone is sufficient to act as a chemiosmotic secondary transport system for arsenite resistance without the presence of an ArsA ATPase [7]. An additional gene, *arsC,* has been shown to encode for an arsenate reductase that mediates reduction of arsenate prior to arsenite efflux [15].

In New Zealand, elevated levels of arsenic are common in geothermal effluent generated from geothermal power stations, which introduce approximately 190 tons of arsenic (75% of total) into the Waikato River [1]. Other sites of high arsenic contamination are those from

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mine tailings generated from historic gold mining activities. For example, the historic Barewood mine, southeast of Middlemarch in Central Otago, contains 100-year-old mine tailings that include the mineral arsenopyrite. The level of dissolved arsenic is generally less than 1 ppm, but there is approximately 100 ppm in surrounding tussock grasslands and 1000 ppm in the wetlands [9].

The aim of the present study was to isolate bacteria from these arsenic-contaminated sites that could either use arsenite as an electron donor or arsenate as an electron acceptor under aerobic and anaerobic growth conditions respectively, or were resistant to the growth-inhibitory effects of these arsenic species through various detoxification pathways.

Materials and Methods

Isolation and enumeration by most probable number (MPN) from environmental samples. Six samples (sediment and water) were collected from the Barewood gold field in central Otago, New Zealand. The samples ranged in external pH from 5.9 to 6.6, and the temperature varied from 18° to 22°C. A further six samples (water and sediment) were collected from Lake Ohakuri, which is affected by geothermal effluent. All water and sediment samples had a pH range of 5.0–5.3, and the temperature was 20°C. Each sample (0.1 g of soil or 100 μL of water) was inoculated into different selective media (see below) to estimate bacterial population numbers. After vigorous mixing, 100-fold step serial dilutions to 10^{-8} were performed for each sample. Media were then incubated for 10 days at 28°C. Results for each dilution were recorded as either growth or no growth. MPN in grams [wet weight] was calculated for each sample. The following selective media were used: minimal salts medium (MS) supplemented with 1% (wt/vol) thiosulfate [13] was used for the culture of aerobic chemoheterotrophs; MS supplemented with 5 mM arsenite for aerobic chemoautotrophs able to utilize arsenite as an electron donor for chemolithotrophy; tryptone, yeast extract, and glucose (TYEG) medium [31] for aerobic chemoheterotrophs; sulfate reducer medium containing 5 mM arsenate for anaerobic heterotrophic sulfate reducers [23]; and anaerobic TYEG medium was used for the culture of fermentative organisms.

Bacteria that were resistant to arsenic species were isolated from heterotrophic cultures (TYEG-grown) supplemented with either arsenate (0–100 mM) or arsenite (0–20 mM) from both the Barewood and Lake Ohakuri sites. To establish whether the isolates were potentially utilizing arsenic as an energy source, or whether the rich TYEG medium buffers the organisms from the toxic effects of arsenic, the isolates were grown in minimal medium [26] supplemented with arsenic.

To follow culture growth and conduct bioenergetic measurements, samples were withdrawn aseptically as required, and culture optical density was measured with a Beckman DU-64 spectrophotometer at 600 nm (OD_{600}) (1 cm light-path length). The growth rate constant (k) for the log phase of growth was determined by plotting the $\log_{10}$ of the optical density against time [25]. Cellular protein was determined by the method of Markwell et al. [21]. Experiments were performed in triplicate.

Identification of bacterial isolates based on 16S rRNA and Biolog plates. One hundred microliters of culture was removed and resuspended in 400 μL sodium-EDTA with 5 μL of 50 mg mL^{-1} lysozyme, 5 μL of 20 mg mL^{-1} proteinase K, and 10 μL of 20% SDS. After 30 min incubation, genomic DNA was extracted with phenol, then twice with phenol/chloroform (50/50), and then once with chloroform. DNA was precipitated in 3 M sodium acetate (pH 5.2) and ice-cold ethanol, pelleted by centrifugation, ethanol washed, and then resuspended in 50 μL TE buffer.

DNA primers were designed to flank the relatively variable region of the 16S rRNA gene [17]. The forward primer, PCR A, covers positions 774 to 795 (*E. coli* numbering system) (5′-GGAGCAAA-CAGGATTAGATACC-3′). The reverse primer, PCR B, covers positions 1419 to 1403 (5′-TGCCAACTCTATGGTGTGTGACG-3′). The positive control was a non-pathogenic *Clostridia* species NCP262. The Biolog system was used to support the 16S rRNA genus identification of isolates. All protocols for preparation and identification of microorganisms are outlined in the Biolog™.

Detection of *arsB* and *arsC* genes and determination of arsenate and arsenite transformation. Primers used for *arsB* amplification were as follows: *arsBF* 5′-ATGGCAACCGAAAGGTTTAG-3′ and *arsBR* 5′-GTTGGCATGTTGTTCATAAT-3′. For *arsC,* the primers were as follows: *arsCF* 5′-AACAGTTGCCGCAGCATTCT-3′ and *arsCR* 5′-ATGCGCTCCAGCTCACGCTT-3′. A wild-type *E. coli* strain isolated in this study was used as a positive control for both *arsB* and *arsC,* and the PCR products from this organism were used as probes for Southern hybridization by using standard molecular biology protocols [27]. PCRs were performed in 50 μL volumes using 1 U Taq DNA polymerase (Roche Molecular Biochemicals, Mannheim, Germany), in accordance with the manufacturer's instructions. Amplification consisted of one cycle at 94°C for 2 min and 28 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, and a final cycle at 94°C for 1 min, at 55°C for 1 min, and at 72°C for 5 min.

The speciation and concentration of arsenate and arsenite were measured with a spectrophotometric method described by Johnson and Pilson [16]. New plasticware was used to avoid contamination from excess phosphate from detergents. For the assay, 1.2 mL of each sample was pipetted into each of six tubes. Two tubes were oxidized, two were untreated, and two were reduced (e.g., duplicates of each for each sample). The absorbance was measured at 865 nm. Blanks of milli-Q water were used to calibrate the spectrophotometer. Standard curves were prepared for concentrations of 0–100 μM for both arsenate and arsenite. The experiment was performed in triplicate to record the standard error of the mean.

Oxygen consumption. For oxygen consumption measurements, cells were harvested from exponentially growing cultures by centrifugation (8000 *g,* 15 min, 4°C), washed in 100 mM sodium-phosphate buffer (pH 7.0) containing 5 mM $MgCl_2$, and resuspended in the same buffer to give protein concentrations of 5–10 mg protein · mL^{-1}. Respiration rates were measured in a Rank Bros. Clark-type oxygen electrode at 35°C as previously described [6]. Arsenite (10 mM) was added as a potential electron donor. The oxygen electrode was calibrated with air-saturated sodium-phosphate buffer. Protein from NaOH-hydrolyzed cells (0.2 M NaOH, 100°C, 20 min) was assayed by the method of Markwell et al. [21].

ArsC enzyme assay. Cell-free crude extracts were made of *Aeromonas* sp. CA1, *Exiguobacterium* sp. WK6, and *Escherichia* sp. CA5 to act as a positive control. Cells were grown to stationary phase in 500 mL of TYEG medium supplemented with 20 mM of arsenate, harvested by centrifugation for 10 min at 7500 *g,* washed twice in 50 mL of reaction buffer (10 mM Tris, pH 7.5, with 1 mM Na_2EDTA and 1 mM $MgCl_2$), and finally resuspended in 15 mL of reaction buffer. Cells were then disrupted by sonication, and unbroken cells were removed by centrifugation at 8000 *g* for 15 min at 4°C. ArsC activity was measured by two methods. The first was an adaptation of the method described by Ji and Silver [15]. Arsenate reduction at 37°C was initiated by mixing 50

μL of crude extract in 180 μL of reaction buffer in a microtiter plate, then adding 12.5 μL of 2 mM arsenate (final concentration 100 μM) and 7.5 μL of 10 mM DTT (final concentration of 300 μM DTT). Adding 50 μL of mixed reagent (as prepared for the arsenic speciation assay described above) stopped the reaction. The amount of arsenate reduced was measured by a decrease in blue arseno-molybdate formation (phospho-molybdate remains constant) by using a Labsystems Multiskan Ascent plate reader at 750 nm.

The second method was based on NADPH oxidation, which is coupled to ArsC reductase activity. NADPH oxidation was initiated at 37°C by mixing 50 μL of crude extract in 820 μL of reaction buffer, 30 μL of 10 mM DTT (final concentration 300 μM), 50 μL of 2 mM arsenate (final concentration 100 μM), and 50 μL of 3 mM NADPH (final concentration 0.15 mM). Arsenate concentrations of 500 μM and 1 mM were assayed along with the same concentrations of arsenite and 'no arsenic' for controls. Measurements were recorded at 340 nm, where 0.15 mM NADPH has an absorbance of approximately 1.0. Absorbance decreases as NADPH is oxidized coupled to arsenate reduction to arsenite. Endogenous NADPH oxidation rates were subtracted from arsenate-induced NADPH oxidation.

Results

Most probable number of bacterial isolates from arsenic-contaminated environments. Enumeration of bacterial populations by using MPN revealed that all Barewood sites and Lake Ohakuri sites had $> 10^6$ aerobic chemoheterotrophs per gram of sediment [wet wt] and $> 10^2$ chemoautotrophs (thiosulfate oxidizers) per gram of sediment [wet wt]. No chemoautotrophs were isolated that could utilize arsenite as an electron donor for chemolithotrophy. All Barewood samples contained fermentative organisms ($> 10^5$ per gram of sediment [wet wt]), but only three sites had culturable sulfate reducers present ($> 10^3$ per gram of sediment [wet wt]). No bacteria were isolated that could use arsenate as an electron acceptor under anaerobic conditions (data not shown). Lake Ohakuri samples were not analyzed for the presence of anaerobic bacteria.

Isolation of arsenic-resistant heterotrophs. Because no bacteria were isolated that were able to use arsenic compounds as electron donors or acceptors, we attempted to isolate bacteria from these sites that were able to tolerate high concentrations of arsenic species (e.g., 0–100 mM arsenate or 0–20 mM arsenite) and perhaps transform these compounds via a novel mechanism. In total, 10 isolates were obtained from the Barewood gold field samples by enrichment and isolation of pure colonies on plates containing high concentrations of the same arsenic species on which the organisms were originally isolated (Table 1). These strains were designated CA1 to CA10. A further seven isolates exhibiting unique colony morphologies were obtained from Lake Ohakuri samples. These isolates were designated WK1 to WK7. Strain identification based on Biolog and 16S rRNA analyses are presented in Table 1.

Table 1. Phylogenetic characterization of heterotrophic isolates from the Barewood gold field and Lake Ohakuri by using the 16S rRNA gene and Biolog genus identification

Isolate name	Arsenic species isolated on	16S rRNA genus identification	Biolog genus identification
CA1	As V	*Aeromonas*	*Aeromonas*
CA2	As V	*Pseudomonas*	*Pseudomonas*
CA3	As V	*Bacillus*	*Bacillus*
CA4	As V	*Bacillus*	Not tested
CA5	As V	*Escherichia*	*Escherichia*
CA6	As V	*Escherichia*	Not tested
CA7	As V	*Pseudomonas*	*Pseudomonas*
CA8	As III	*Acinetobacter*	*Acinetobacter*
CA9	As III	*Pseudomonas*	*Pseudomonas*
CA10	As III	*Pseudomonas*	Not tested
WK1	As V	*Bacillus*	Not tested
WK2	As V	*Bacillus*	Not tested
WK3	As V	*Bacillus*	*Bacillus*
WK4	As V	*Pseudomonas*	Not tested
WK5	As V	*Pseudomonas*	*Pseudomonas*
WK6	As V	*Exiguobacterium*	Not in database
WK7	As III	*Bacillus*	*Bacillus*

The following strains were chosen for further study; *Aeromonas* sp. CA1, *Pseudomonas* sp. CA2, *Bacillus* sp. CA3, *Pseudomonas* sp. CA7, *Bacillus* sp. WK3, *Pseudomonas* sp. WK5, and *Exiguobacterium* sp. WK6. These strains were grown in TYEG containing arsenate at varying concentrations from 0 to 100 mM. Of these seven isolates, strain WK6 exhibited a significant increase in the final OD_{600} as the external arsenate concentration increased from 0 to 80 mM, suggesting that strain WK6 was gaining an energetic advantage from arsenate (Fig. 1A). For strain WK6, the growth rate in the absence of arsenate was 0.67 $h^{-1} \pm 0.19$ (doubling time of 1.04 h) and 0.97 $h^{-1} \pm 0.11$ (doubling time of 0.72 h) in the presence of 50 mM arsenate (data not shown). Arsenate at concentrations up to 30 mM also had a stimulatory effect on the growth rate and a final cell yield of isolate CA1 (Fig. 1B). All other isolates with the exception of CA2, which was completely resistant to all arsenate concentrations tested, showed a decrease in the final optical density with increasing arsenate concentration (Fig. 1B).

To further substantiate the claim that these bacteria were indeed able to gain metabolic energy from arsenate and arsenite, total cellular protein was determined to provide direct evidence for an increase in cell biomass in the presence of arsenite or arsenate. Based on this method, the total cell yield was approximately twofold higher in the presence of 30 mM and 50 mM arsenate respectively for both isolates CA1 and WK6 (Fig. 1C).

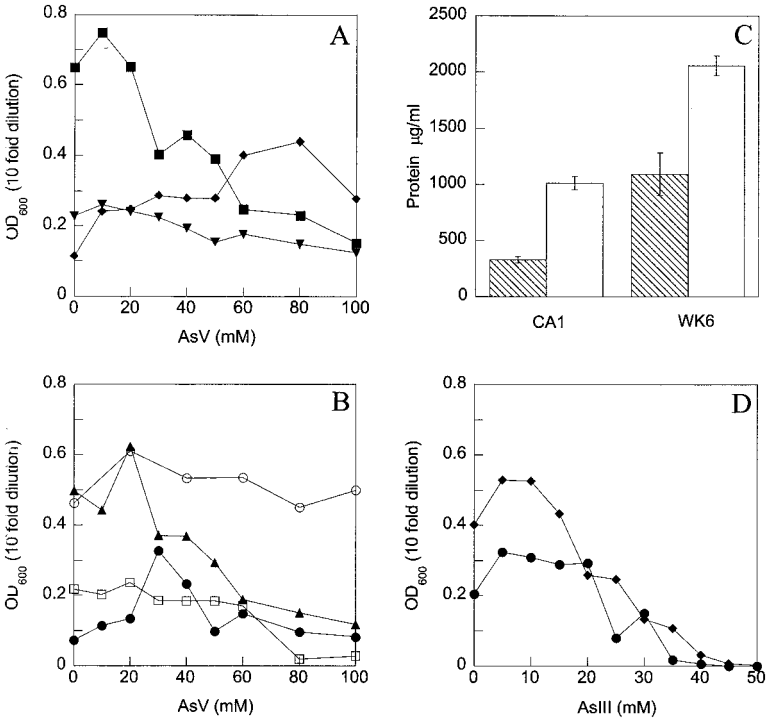

Fig. 1. Growth of isolates from Lake Ohakuri (A) and Barewood gold field (B) in arsenate (0–100 mM)-containing medium. Isolates WK3 (▼), WK5 (■), and WK6 (◆) (A). Isolates CA1 (●), CA2 (○), CA3 (□), and CA7 (▲) (B). The final OD_{600} readings were recorded after 24 h growth, and the results shown are the mean of three experiments. (C) Cellular protein yields for isolates CA1 and WK6 grown on 30 mM and 50 mM arsenate, respectively (open bars). Control cultures with no arsenate are shown (shaded bars). Cells were harvested and assayed 24 h after inoculation. Error bars represent the standard error of the mean. (D) Growth of isolates CA1 (●) and WK6 (◆) on increasing concentrations of arsenite (0–50 mM).

Based on these results, the final OD_{600} was a reliable and accurate representation of cell yield.

Identical experiments were performed with arsenite. Two strains, CA1 and WK6, showed an increase in growth yield with increasing arsenite concentration up to 5–10 mM as determined by final optical density (Fig. 1D). Arsenite (10–15 mM) stimulated the growth rate of strain CA1 1.56-fold and strain WK6 by 1.26-fold (data not shown). No growth stimulation of either isolate CA1 or WK6 by arsenate or arsenite was observed in minimal medium containing glucose, suggesting that the growth stimulation was unique to rich medium (data not shown).

The increase in growth yield and growth rate in the presence of arsenate and arsenite for strains WK6 and CA1 was not easy to rationalize in terms of conventional bioenergetic schemes. We tested the ability of these cells to use the more reduced species arsenite as an electron donor. Washed cell suspensions of strain CA1 and WK6 grown in the presence of arsenite were resuspended in 100 mM K-phosphate buffer (pH 7.0) and respiration measured by using an O_2 electrode. Under no conditions tested could arsenite stimulate respiration of strain CA1 or WK6, demonstrating that arsenite was not used as an electron donor (data not shown).

Reduction of arsenate to arsenite by isolates CA1 and WK6 is coupled to alkalinization of the growth medium. During growth of strain CA1 in rich medium, it was observed that growth slowed as the external pH approached pH 6.0 or below (data not shown). A pH profile for isolates CA1 and WK6 revealed that the optimum pH for growth was pH 8.0 to 9.0 under pH-uncontrolled batch culture (Fig. 2). The final pH was consistently between pH 5.5 to 6.0.

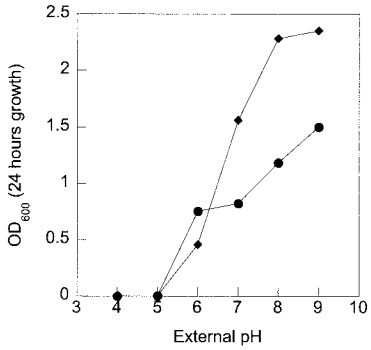

Fig. 2. Growth of isolates CA1 (●) and WK6 (◆) over the pH range 5–9 with no added arsenate. The initial pH of the culture is shown, and the final OD_{600} was measured 24 h after inoculation. Results shown are the mean of three experiments.

When isolate CA1 was grown in 10 mM arsenite, less than 2 mM arsenate could be detected in the growth medium, but importantly no significant conversion of arsenite to arsenate was noted (Fig. 3A and 3B). The final pH of the growth medium was < 5.5. When isolate CA1 was grown with 50 mM arsenate, arsenate was reduced to arsenite during exponential growth until the arsenite concentration reached around

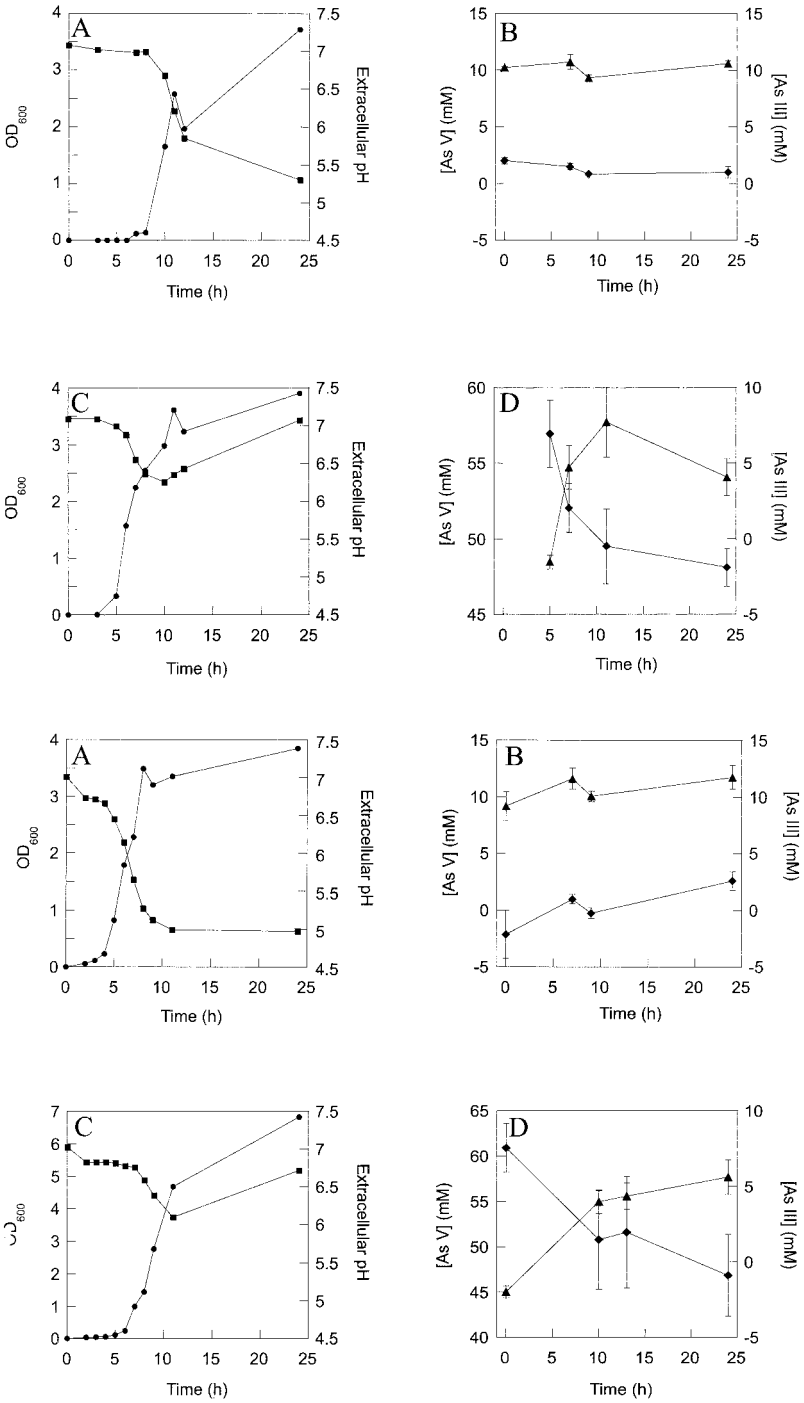

Fig. 3. (A and B) Growth of isolate CA1 in the presence of 10 mM arsenite (As III) for 24 h. Change in OD_{600} (●) versus extracellular pH (■) over 24 h (A) and concentrations of As III (▲) and As V (◆) over the time course of the experiment (B). (C and D) Growth of isolate CA1 in the presence of 50 mM arsenate (As V) for 24 h. Change in OD_{600} (●) versus extracellular pH (■) over 24 h (C) and concentrations of As V (◆) and As III (▲) over the time course of the experiment (D). Error bars indicate the standard error of the mean. The initial starting pH in these experiments was 7.

Fig. 4. (A and B) Growth of isolate WK6 in the presence of 10 mM arsenite (As III) for 24 h. Change in OD_{600} (●) versus extracellular pH (■) over 24 h (A) and concentrations of As III (▲) and As V (◆) over the time course of the experiment (B). (C and D) Growth of isolate WK6 in the presence of 50 mM arsenate (As V) for 24 h. Change in OD_{600} (●) versus extracellular pH (■) over 24 h (C) and concentrations of As V (◆) and As III (▲) over the time course of the experiment (D). Error bars indicate the standard error of the mean. The initial starting pH in these experiments was 7.

4 mM in the growth medium (Fig. 3D). Under these conditions, the final pH of the growth medium was 7.0 (Fig. 3C), suggesting that the stimulation of growth by arsenate was due to the lack of medium acidification. An identical pattern of arsenite and arsenate conversion was observed with strain WK6 (Fig. 4). Arsenite was not transformed by isolate WK6, and arsenate was reduced to arsenite with concomitant alkalinization of the growth medium.

To elucidate the pathway of arsenate reduction in isolates CA1 and WK6, amplification of the *ars* operon in isolates WK6 and CA1 was carried out. Only for isolate WK6 was a positive PCR product for the *arsB* gene obtained. This was also confirmed by Southern

hybridization of the *arsB* genes to strain WK6 chromosomal DNA (data not shown). Isolate CA1 was not positive for *arsB* based on PCR and Southern hybridization. The *arsC* gene could not be detected in either isolate via PCR and Southern hybridization (data not shown). No ArsC enzymatic reduction of arsenate could be detected in isolates CA1 and WK6 (data not shown).

Discussion

Recent studies have described the isolation and characterization of arsenite-oxidizing bacteria from Australian gold mining environments able to grow chemolithotrophically with oxygen as an electron acceptor and arsenite as the electron donor [28]. Bacteria able to utilize arsenate as an alternative electron acceptor anaerobically have also been recovered from these environments [2, 20, 23, 24, 28–30]. The initial aims of this study were to isolate microorganisms from arsenic-contaminated sites (e.g., gold mining and geothermal effluent) that could use arsenic compounds for either anaerobic respiration (e.g., arsenate) or as electron donors (e.g., arsenite) for chemolithotrophy. No bacteria were recovered that could use arsenic compounds under these conditions. However, several bacterial isolates were recovered that could grow in the presence of high concentrations of arsenic and arsenate. Of the genera isolated in this study, *Aeromonas, Exiguobacterium,* and *Acinetobacter* have not been previously isolated from arsenic-contaminated sites. Both *Aeromonas* and *Exiguobacterium* have not been previously known to have direct interactions with arsenic species. Phylogenetically, these bacteria are distantly related, being from the gamma and delta proteobacteria and the high GC Gram-positive bacteria.

Both isolates WK6 and CA1 were able to transform arsenate to arsenite, but no transformation of arsenite could be detected. Of the heterotrophic bacteria discovered so far that can transform arsenate, only non-respiratory arsenate reductases in *Escherichia coli* and *Staphylococcus aureus* have been studied in detail. Arsenate reduction in these bacteria is catalyzed via the *ars* operon encoding an arsenate reductase (*arsC*) and an arsenite efflux pump (*arsB*) [7]. Arsenate reduction is considered a method of detoxification, even though the arsenite is more toxic than arsenate. Currently, *Chrysiogenes arsenatis* is the only bacterium that is characterized biochemically to respire anaerobically by using arsenate as a terminal electron acceptor and acetate as the electron donor [18]. During growth, the arsenate is reduced to arsenite; the reduction is catalyzed by an arsenate reductase similar to a number of prokaryotic molybdenum-containing polypeptides (e.g., the formate dehydrogenases H and N of *Escherichia coli*). In the present study, isolates WK6 and CA1 were able to convert arsenate to arsenite. Isolate WK6 was positive for *arsB* but negative for *arsC,* and therefore an *arsC*-independent pathway may exist for the reduction of arsenate. Isolate CA1 was negative for the presence of *arsB* and *arsC,* and therefore arsenate reduction may be via a novel pathway, or the *ars* operon in this bacterium has low similarity with previously published operons, and therefore neither PCR nor Southern hybridization would detect the presence of this operon. In this respect, it has been reported that the arrangement of the *ars* operons in different bacterial genera can vary [5].

Under certain conditions, the conversion of arsenate to arsenite uses excess hydrogen ions [20]. In experiments on an arsenate-respiring bacterium, Macy et al. [20] reported that the external pH increased to 9.4 when the organisms were using acetate as an electron donor coupled to arsenate reduction. Although the isolates in this study did not couple arsenate reduction to substrate oxidation, arsenate reduction did cause significant alkalinization of the growth medium. This extracellular alkalinization of the growth medium prevented the external pH from decreasing to a point where it inhibited growth, resulting in an increased cellular growth yield and growth rate under these conditions for isolates CA1 and WK6. Species of *Exiguobacterium* and *Aeromonas* are known to be alkaliphilic [8, 19]; therefore, arsenate reduction acts to buffer medium acidification, favoring the growth of these bacteria.

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