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Arsenite-Oxidizing *Hydrogenobaculum* Strain Isolated from an Acid-Sulfate-Chloride Geothermal Spring in Yellowstone National Park

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An arsenite-oxidizing *Hydrogenobaculum* strain was isolated from a geothermal spring in Yellowstone National Park, Wyo., that was previously shown to contain microbial populations engaged in arsenite oxidation. The isolate was sensitive to both arsenite and arsenate and behaved as an obligate chemolithoautotroph that used H_2 as its sole energy source and had an optimum temperature of 55 to 60°C and an optimum pH of 3.0. The arsenite oxidation in this organism displayed saturation kinetics and was strongly inhibited by H_2S.

Arsenite [As(III)] is often the predominant valence of inorganic arsenic in geothermal source waters, although arsenate [As(V)] can also be present, with As(V)/As(III) ratios varying among different springs due to mixing with meteoric surface waters prior to discharge (3, 12, 19). However, subsequent to discharge, As(V)/As(III) ratios in the spring water can also be significantly influenced by redox transformations (10, 12), which are well documented for microorganisms (2, 4, 5, 6, 10, 11, 16, 17). As(V) reduction is widespread among prokaryotes, occurring when As(V) is utilized as an electron acceptor for anaerobic or microaerobic respiration (13) or as part of a detoxification strategy (8). As(III) oxidation has likewise been observed in various organisms, where it has also been viewed as an apparent detoxification mechanism (1, 14, 15) or as a source of energy to support chemolithoautotrophic growth (1, 17).

We previously documented rapid microbial oxidation of As(III) in an acid-sulfate-chloride-type geothermal spring in Norris Geyser Basin, Yellowstone National Park (9). This shallow spring is fed by a nearly constant geothermal source water (63°C, pH 3.1) containing ~35 μM As(III). The prokaryote microbial community in this spring forms visually and chemically distinguishable mats. A filamentous yellow microbial mat containing visible amounts of S^0 (63 to 60°C) is present 0 to ~3.5 m from the spring source and changes to a brown, Fe(III) oxyhydroxide filamentous microbial mat (51 to 55°C) at ~3.5 to 5 m from the spring source (9). Chemical analysis of the aqueous and solid phases documented high rates of As(III) oxidation in the brown mat region, and the role of microorganisms in As(III) oxidation was confirmed in assays that showed no As(III) oxidation in the formaldehyde-killed samples (9). The PCR-generated 16S ribosomal DNA clone libraries representing the yellow and brown mat regions were dominated by *Hydrogenobaculum*- and *Desulfurella*-like sequences (7). However, since the phylogenetic data could not predict which population(s) was involved in the As(III) oxidation, the present study was conducted to initiate isolation and characterization of the As(III)-oxidizing microorganism(s) in this spring for use in modeling important and dominant biogeochemical features found in this spring type.

Sampling, enrichment, and isolation. Brown microbial mat material was aseptically sampled and transferred to sterile 70-ml serum bottles and submerged with 35 ml of spring water sampled from above the mat. The bottles were sealed on site with sterile butyl rubber stoppers and transported to the laboratory. The phylogenetic information from community clone libraries (7) indicated that H_2 metabolism was potentially important in the resident community and guided the initial headspace gas treatments, which included (i) filter-sterilized air, (ii) filter-sterilized air enriched with 10% CO_2, (iii) filter-sterilized gaseous N_2 [N_2(g)] for anaerobic incubations, and (iv) filter-sterilized 85% H_2(g) plus 10% CO_2(g) plus 5% air. Killed controls (4% formaldehyde) were included, and all bottles were amended with 50 μM As(III) (as $NaAsO_2$) and incubated at 55°C. After 7 days of incubation, As(III) oxidation was measured using hydride generation atomic absorption spectrophotometry (see reference 9) and found to occur only in those treatments containing H_2(g). Denaturing gradient gel electrophoresis (DGGE) (described previously [7]) was used to assess enrichment progress and showed that the microorganisms in the H_2(g) treatment appeared to be a subset of the original microbial community (Fig. 1, compare lanes 1 and 2).

The microaerobic and H_2 requirements for As(III) oxidation suggested that *Hydrogenobaculum*-like organisms may be important, and therefore subsequent enrichment steps employed a synthetic medium used previously for the isolation of *Hydrogenobaculum* (18). No growth was obtained on medium solidified with Phytagel, and therefore progress towards a pure culture was monitored via phase-contrast microscopy and DGGE. Following several dilution-extinction enrichment steps and two cycles of dilutions to a theoretical 0.5 cell/ml (based on 4′,6′-diamidino-2-phenylindole dihydrochloride

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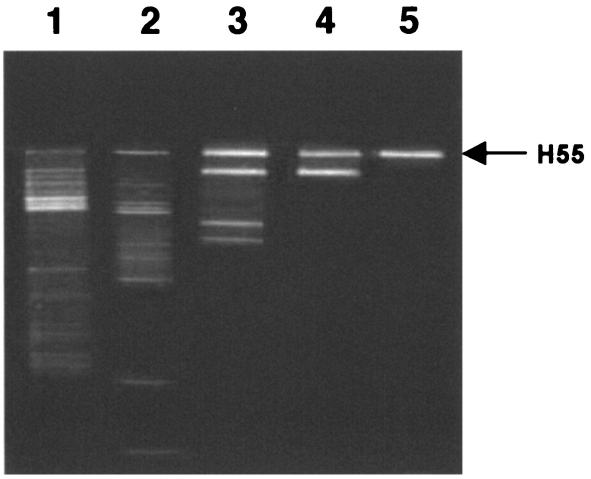

FIG. 1. Documentation of enrichment and isolation of *Hydrogenobaculum* sp. strain H55. PCR-DGGE analysis of 16S ribosomal DNA fragments derived from organisms present in the original mat sample (lane 1), after initial H_2 gas headspace enrichment (lane 2), and in subsequent culture transfers with continued selection under H_2 and microaerobic conditions and with screening for As(III) oxidation (lane 3, third transfer; lane 4, sixth transfer) and of the eventual pure culture *Hydrogenobaculum* sp. strain H55 (lane 5). DNA was extracted and PCR amplified by methods previously described (7).

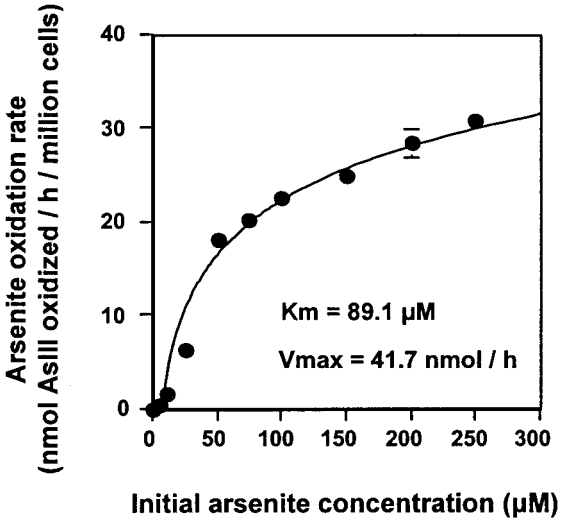

FIG. 2. H55 As(III) oxidation kinetics. Shown are As(III) oxidation rates (●) as a function of initial As(III) concentration. Rate data were fit to the Michaelis-Menten equation by using nonlinear regression (shown by the line) to estimate the kinetic parameters K_m and V_{max}. Rates are the mean changes in As(V) concentration measured in 1-ml assay volumes (1.0×10^6 cells · ml^{-1}) from triplicate cultures, and error bars (where visible) represent 1 standard error of the mean.

[DAPI]-stained cells and direct microscopic counts), the uniform cell morphology correlated with a stepwise reduction in DGGE profile complexity (Fig. 1). An As(III)-oxidizing culture was verified to represent a single isolate by showing that sequences of 10 independent clones from the purified DGGE band (Fig. 1, lane 5) were identical. The organism was referred to as isolate H55.

Isolate characterization. The nearly full-length 16S rRNA gene of H55 was PCR cloned and sequenced (GenBank accession no. AY268103) by previously described protocols (7). BLAST analysis determined the closest match to be *Hydrogenobaculum acidophilum* (98% identical to accession no. D16296). The incubation temperature and medium pH were varied in order to establish the optimum temperature and pH for H55 as being 55 to 60°C and 3.0, respectively, which are in close agreement with the corresponding values for the environment from which H55 was isolated (55°C and pH 3.1). Under optimum conditions, the H55 doubling time was approximately 25 h. No growth was observed with various carbon compounds (including formate and acetate) in a defined medium or in complex media containing yeast extract or tryptone, suggesting that H55 may be an obligate autotroph.

Filtrates of mature H55 cultures did not oxidize As(III) (results not shown), indicating that the oxidation activity was intimately associated with the cell. Kinetic parameters were estimated for whole cells by substrate saturation assays initiated by suspending washed cells (1.0×10^6 per ml) in the synthetic growth medium amended with various As(III) concentrations. Linear plots of As(III) oxidation versus time determined As(III) oxidation rates, which were then plotted against substrate concentrations and analyzed via nonlinear regression to calculate values for K_m and V_{max} based on the Michaelis-Menten equation (Fig. 2).

H55 tolerance of As(III) and As(V) was investigated in the presence of 2.5 mM phosphate. Under these conditions, H55 failed to grow in arsenic concentrations above 1 mM (Fig. 3A) and was slightly more sensitive to As(V) than to As(III). Phosphate concentration and corresponding P/As ratios influenced As toxicity (the minimum phosphate concentration required for maximum growth was 0.75 mM). As expected, As(III) sensitivity was independent of P concentrations ranging from 0.5 to 5 mM, whereas As(V) sensitivity was inversely correlated with phosphate, a chemical analog of As(V) (Fig. 3B).

Effects of Fe(III) and H_2S on As(III) oxidation. In previous work with this spring, it was found that As(III) oxidation did not occur in the absence of live microorganisms, even if significant Fe oxyhydroxide solid-phase mineral material was present in the sediments used in the assays (9). Therefore, additional experiments in the present study were conducted to determine whether dissolved Fe(III) could contribute significantly to As(III) oxidation and whether Fe(III)-catalyzed As(III) oxidation rates compared favorably to those measured with H55. Fe(III) (as $FeCl_2$) and As(III) were added to triplicate inoculated and noninoculated serum bottles at 50 μM each; this Fe concentration approximates spring levels but assumes that all aqueous Fe occurs as Fe(III). No As(III) oxidation occurred in the noninoculated controls with or without Fe(III), whereas roughly 70% of the As(III) was oxidized in the inoculated treatments (7 days of incubation at 55°C and pH 3.5 [results not shown]).

Other previous work at this thermal spring had also found that As(III) oxidation was not measurable where aqueous sulfide levels ranged from 60 to 80 μM (12), even though *Hydrogenobaculum* populations were present (7). Therefore, the effect of aqueous sulfide on As(III) oxidation by the H55 isolate was investigated by adding 60 μM H_2S and 30 μM As(III) to

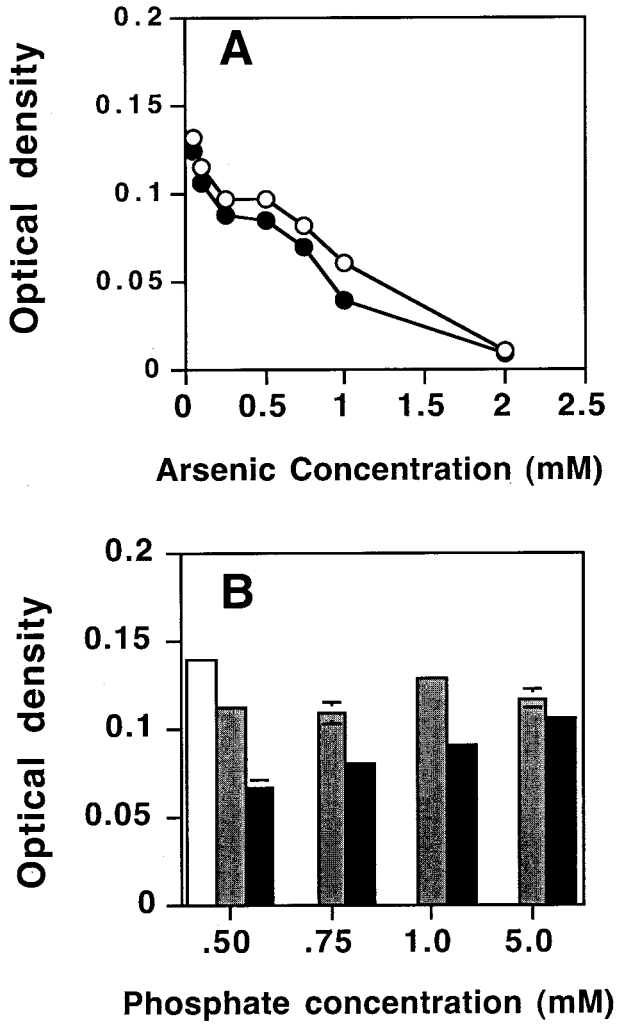

FIG. 3. Sensitivity of *Hydrogenobaculum* sp. strain H55 to arsenic. (A) Growth response of *Hydrogenobaculum* strain H55 as a function of increasing concentrations of As(V) (●) or As(III) (○). (B) Effects of various phosphate concentrations on the growth of H55 in the presence of 0.75 mM As(III) (shaded bar) or 0.75 mM As(V) (filled bar) compared to growth occurring in the absence of As (open bar). Cultures were inoculated to an initial optical density (measured at A_{595}) of 0.02. Results in both panels are from one of two independent experiments demonstrating similar responses. Each value is the mean of results for three replicate cultures for each As concentration. Error bars (where visible) depict 1 standard error of the mean.

H55 cultures to mimic measured concentrations in the spring (9). Normal As(III) oxidation patterns were observed in cultures not treated with H_2S, but the presence of H_2S inhibited As(III) oxidation (Fig. 4). Furthermore, the addition of sulfide as a spike midway through the experiment correlated with an immediate arrest of As(III) oxidation (Fig. 4). Noninoculated controls failed to show any As(III) oxidation or any reduction of As(V) due to H_2S at the concentrations used (Fig. 4). We also note that the negative effect of sulfide on As(III) oxidation was not correlated with cell viability since the growth of H55 was not inhibited by 60 μM sulfide (results not shown).

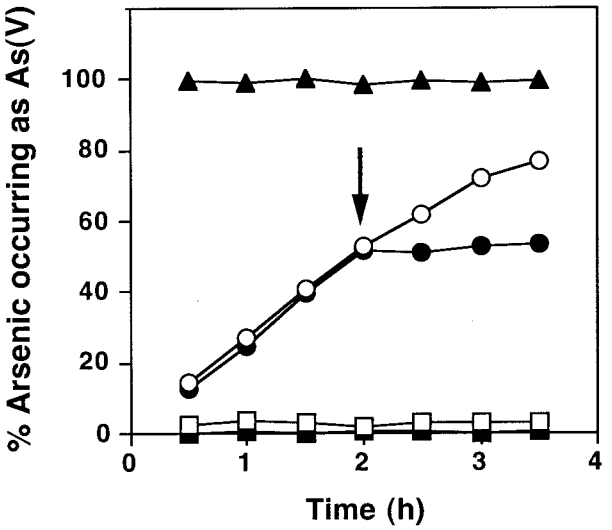

FIG. 4. Influence of aqueous sulfide on As(III) oxidation by *Hydrogenobaculum* sp. strain H55. Levels of As(III) oxidation in cultures (1.0×10^6 cells · ml^{-1}, 5% air, 55°C, pH 3.0) where sulfide was omitted (○) or added at the beginning of the experiment (□) or as a spike (indicated by arrow) after 2 h of incubation (●) are shown. Additional controls included noninoculated medium containing 30 μM As(III) (■) or 30 μM As(V) (as $Na_2HAsO_4 \cdot 7H_2O$) plus 60 μM S(−II) (as Na_2S) (▲). Equilibrium equations were used to calculate and correct for the partitioning of aqueous H_2S and H_2S(g) in the serum bottle headspace. Culture samples were taken every 0.5 h for As speciation and analysis as described previously (9). Results are from one of two independent experiments demonstrating this response, where each data point is the mean of results of three replicate samples. Error bars (not visible) depict 1 standard error of the mean.

Summary. The isolation and characterization experiments with *Hydrogenobaculum* sp. strain H55 were targeted at identifying the organism(s) involved in arsenite oxidation in the native spring and to begin to establish the components of a conceptual model that will help explain the important microbial processes that influence the biogeochemistry in this type of geothermal spring. As(III) oxidation was detected only in those treatments that involved microaerobic levels of oxygen (~1% O_2) with CO_2 as the primary carbon source and H_2 as the primary energy source. These conditions were expected to favor the *Hydrogenobaculum*-like organisms whose presence in this spring were previously documented (7). Also, the temperature and pH optima of the isolate corresponded to values for in situ conditions (9) and serve as minimum points of relevancy that link this organism to the environment and biogeochemistry being studied. The maximum rate of As(III) oxidation by isolate H55 was estimated to be approximately 0.7×10^{-9} μmol · $cell^{-1}$ · min^{-1} (Fig. 2), but at typical in situ As(III) concentrations, As(III) oxidation rates might approach 2.2×10^{-10} μmol · $cell^{-1}$ · min^{-1}. Based on previous estimates of total As(III) oxidation at this site (0.2 μmol of AsIII oxidized · min^{-1} · cm of $sediment^{-3}$; calculated from data in reference 9), an H55 population density of approximately 9×10^8 cells · cm of $sediment^{-3}$ would be necessary to account for all of the As(III) oxidization. However, the many other *Hydrogenobaculum*-like populations that inhabit this spring (7) may also contribute to As(III) oxidation. The physiologic basis for As(III)

oxidation by H55 is unclear. It appears to be unable to utilize As(III) as an energy source for chemolithoautotrophic growth (results not shown), yet a detoxification basis also can be questioned because H55 is more sensitive to As(V).

The addition of dissolved Fe(III) to H55 samples did not influence As(III) oxidation. This finding is consistent with previous in-field assays that found no As(III) oxidation with biocide-treated mat sediment samples (9) containing Fe(III) oxyhydroxides and aqueous Fe [mainly Fe(II)] and suggests that the Fe(III) in this mat environment (either solid phase or dissolved) likely contributes little to measured As(III) oxidation rates, at least relative to those catalyzed by H55 or other similarly behaving microorganisms. The profound negative effect of sulfide on As(III) oxidation by H55 implies that the absence of As(III) oxidation in regions of the spring containing appreciable levels of sulfide (9) may be due to sulfide inhibition of microbial As(III) oxidases. Future studies at this spring will attempt to further refine our understanding of microbe-arsenic interactions and to determine the relative importance of H_2, H_2S, and As(III) as primary energy inputs for the total microbial community.

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