

Relative Importance of H₂ and H₂S as Energy Sources for Primary Production in Geothermal Springs^{∇†}

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Geothermal waters contain numerous potential electron donors capable of supporting chemolithotrophy-based primary production. Thermodynamic predictions of energy yields for specific electron donor and acceptor pairs in such systems are available, although direct assessments of these predictions are rare. This study assessed the relative importance of dissolved H₂ and H₂S as energy sources for the support of chemolithotrophic metabolism in an acidic geothermal spring in Yellowstone National Park. H₂S and H₂ concentration gradients were observed in the outflow channel, and vertical H₂S and O₂ gradients were evident within the microbial mat. H₂S levels and microbial consumption rates were approximately three orders of magnitude greater than those of H₂. *Hydrogenobaculum*-like organisms dominated the bacterial component of the microbial community, and isolates representing three distinct 16S rRNA gene phylotypes (phylotype = 100% identity) were isolated and characterized. Within a phylotype, O₂ requirements varied, as did energy source utilization: some isolates could grow only with H₂S, some only with H₂, while others could utilize either as an energy source. These metabolic phenotypes were consistent with in situ geochemical conditions measured using aqueous chemical analysis and in-field measurements made by using gas chromatography and microelectrodes. Pure-culture experiments with an isolate that could utilize H₂S and H₂ and that represented the dominant phylotype (70% of the PCR clones) showed that H₂S and H₂ were used simultaneously, without evidence of induction or catabolite repression, and at relative rate differences comparable to those measured in ex situ field assays. Under in situ-relevant concentrations, growth of this isolate with H₂S was better than that with H₂. The major conclusions drawn from this study are that phylogeny may not necessarily be reliable for predicting physiology and that H₂S can dominate over H₂ as an energy source in terms of availability, apparent in situ consumption rates, and growth-supporting energy.

Thermophiles dominate the deepest and shortest branches of the *Bacteria* and *Archaea* domains in the tree of life, suggesting that they are likely ancestors of Earth's contemporary microbial populations (8, 35). Consequently, these organisms have attracted considerable attention due to interest in the origin of enzymes and metabolic pathways that are thought to have evolved from such organisms. Chemolithotrophic metabolism is foundational to primary productivity in geothermal environments where temperatures exceed the limit of photosynthesis. The bioenergetics of such systems have been examined from the perspective of theoretical energy yield as a way of discussing the relative importance of the various electron donors and acceptors that could support primary productivity (3–5, 22). Other studies have sought to link the inferred physiology of microbial populations with the predicted energy yields obtainable from the inorganic constituents present (4, 18, 26, 28, 33).

Geothermal features vary considerably with respect to temperature, pH, and the presence and concentrations of energy sources such as H₂, H₂S, S⁰, Fe(II) and As(III) (18, 29). Acid-sulfate-chloride (ASC) springs (e.g., Fig. 1) are common throughout the Yellowstone geothermal complex, although they are considerably more concentrated in and around the Norris Geyser Basin area. ASC springs are intriguing from a bioenergetics standpoint because they offer a virtual buffet of energy sources for chemolithoautotrophs, including a constant flux of μM concentrations of dissolved H₂S, Fe(II), and As(III), nM concentrations of H₂, conspicuous amounts of S⁰, and often-supersaturating levels of CO₂ (10, 18, 21, 26, 37). Discussions regarding the relative importance of these electron donors in supporting primary production in such systems sometimes center on comparisons of potential energy released from their oxidation, with the H₂/O₂ couple perhaps being favored (37), whereas others urge caution in making such predictions, pointing out that in low-pH systems, the oxidation of H₂S could yield nearly as much energy (18).

ASC springs are clearly in the latter category and are the focus of the current study, in which we examined microbial utilization of H₂S and H₂ by using a combination of in-field ex situ assays, microelectrodes, and gas chromatography to document the presence, concentrations, gradients, and consumption of these energy sources. These analyses were combined with molecular-community analysis and with the cultivation of

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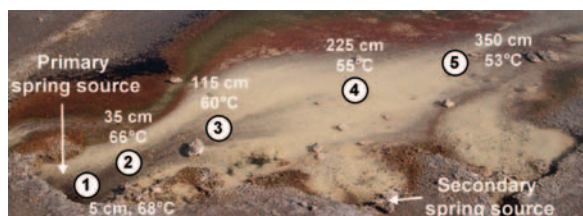


FIG. 1. Color image of Dragon Spring in Norris Geyser Basin. The picture depicts changes in the spring outflow channel structure relative to that in previous studies (10, 21), including additional sources emerging downstream from the main source. Sampling transect sites are numbered (starting at the spring source), and representative temperatures occurring during the present study reflect the thermal gradient.

ecologically relevant organisms that represent the dominant phylotypes. Results from these experiments can be summarized by two important conclusions: (i) microbial phylogeny cannot necessarily be relied upon to predict physiology, and (ii) H₂S can dominate over H₂ in terms of availability, apparent consumption rates, and growth-supporting energy.

MATERIALS AND METHODS

Study site and in situ chemical analysis. Field experiments were conducted at Dragon Spring (44°43'54.8" N, 110°42'39.9" W, spring number NHSP106 in the Yellowstone National Park [YNP] thermal inventory), located in Norris Geyser Basin, YNP. Water temperature and pH were routinely monitored throughout the 2-year study. The pH of the source water was 3.1 on all but two occasions, on which it was found to be 2.8, and the temperatures ranged from 68 to 72°C. Dissolved H₂S was measured by using the methylene blue method (9). Spectrophotometric analysis was performed at springside, using a USB2000 portable spectrophotometer (Ocean Optics, Dunedin, FL). The presence of H₂ was determined by using a portable Varian gas chromatograph (model CP-2900) with Ar and N₂ as carrier gases. The concentration of aqueous H₂ [H₂(aq)] in each sample was determined by using the headspace gas chromatography method and temperature-corrected Henry's law constants described previously (18).

Ex situ measurements of H₂S and H₂ consumption activity. Mat samples were collected by using sterile wide-bore pipette tips and were placed into sterile 15-ml conical tubes, homogenized by mixing, and split into two subsamples. One subsample was maintained at in situ temperature and was used to assay for microbial H₂ or H₂S consumption, while the other was boiled for 20 min to serve as a killed control. Subsamples of live and heat-treated mat material were aseptically transferred into autoclaved 150-ml serum bottles containing 40 ml of filter-sterilized spring water. The assay bottles were immediately closed with rubber septa, pressure sealed with aluminum rings, and incubated at in situ temperatures by placing the bottles in the spring at the same location from which the mat material was sampled. H₂O samples were withdrawn at timed intervals to be assayed for H₂S(aq), whereas headspace gas samples were taken to measure H₂. Data for dissolved H₂S and H₂ levels were normalized based on the mat sample dry weight, which was determined by collecting all mat material from each serum bottle onto preweighed 0.2- μ m filters, drying it overnight at 65°C, and then weighing it. The maximum mat sample dry weight variation between samples was 4.1%.

Cultivation and isolation. Mat samples were collected from sites 35, 115, and 350 cm from the discharge point of the spring. The mat material was stored for transportation in 15-ml conical tubes and maintained at 65°C in a Thermo bottle filled with water from the site. Mat samples were resuspended in 10 ml of spring water collected directly above the mat-sampling site, and 100 μ l of this suspension was used to inoculate 16-ml serum bottles sealed with Teflon-coated butyl rubber septa (National Scientific, Rockwood, TN). For aerobic H₂S chemolithoautotroph enrichment experiments, the serum bottles contained 4.9 ml of filter-sterilized spring water amended with 200 μ M Na₂S and buffered to pH 3.1 with 5 mM citric acid and a headspace containing 50% ambient air and 50% CO₂ for aerobic culture conditions or 90% CO₂ and 10% ambient air for microaerobic conditions. For aerobic H₂ chemolithoautotroph enrichment experiments, the serum bottle setup was the same, except that Na₂S was omitted and headspace gas composition consisted of 50% CO₂, 25% H₂, and 25% ambient air. Microaerobic H₂ enrichments were the same, except that the headspace composition was 50% CO₂, 40% H₂, and 10% ambient air. Enrichment cultures were

incubated at temperatures ranging from 55 to 70°C, corresponding to in situ temperatures where the mat sample inoculum was collected. At monthly intervals, 100 μ l of culture was transferred to similarly prepared serum bottles, and the enrichment cultures were monitored for growth by phase-contrast microscopy. Progress toward a clonal culture was monitored by using denaturing gradient gel electrophoresis (DGGE; see below). After 120 days (i.e., after four enrichment subcultures), cultures were diluted to \sim 0.5 cells \cdot ml⁻¹, with 1.0 ml of this dilution used as inoculum for each of 20 similarly prepared serum bottles. These bottles were monitored for growth as above, and positive cultures (\sim 40% of the bottles) were again diluted and used as inocula for two additional rounds of enrichment initiated from \sim 0.5 cells \cdot ml⁻¹ dilutions. The resulting cultures were considered clonal based on the following criteria: (i) direct sequencing of whole-culture PCR amplicons produced sequencing chromatograms showing no evidence of multiple templates, and (ii) the sequence derived from the whole-culture PCR product exactly matched that of individual sequences derived from a minimum of 20 clones.

DNA extraction, PCR, DGGE, and clone libraries. Total DNA was extracted from enrichment cultures and isolates by using the method of Pitcher et al. (31). Nearly full-length 16S rRNA genes were amplified and cloned, using PCR primers and protocols we have previously described (11, 19). Sequencing for these clones as well as all other clones (below) was carried out at the Ohio State University Plant Microbe Genomics Facility. DGGE analysis of enrichment cultures was also carried out as previously described (11). Internally transcribed spacer (ITS) regions were amplified by using the primers and cycling conditions previously described (14).

16S rDNA clone libraries were constructed from DNA extracted (6) from mat material taken from the Dragon Spring S⁰ deposition zone (yellow area in Fig. 1) at sites 35 and 115 cm from the point of discharge. Nearly full-length 16S rRNA genes were amplified as described above and cloned into pCR2.1 (Invitrogen, Carlsbad, CA), using the manufacturer's suggested protocols. For each site, 45 clones were sequenced. Sequences were aligned by using ClustalX (40) and were edited and trimmed by using Se-Al v2.0a11 (<http://tree.bio.ed.ac.uk/software/seal/>). Phylogenetic analysis of the aligned sequences was performed, using the maximum likelihood algorithm, and bootstrap values for 100 pseudoreplicates were generated by using the PAUP* v4.0b10 software package (39). *Hydrogenothermus marinus* (GenBank accession number AJ292525.1), a member of the family Aquificaceae, was used as the out-group.

Pure-culture studies. An isolate representative of the major 16S rRNA gene phylotype that was recovered from the spring was used for studies that compared growth on H₂S and H₂. Serum bottles were set up as described above, with cell growth rates on these substrates determined by phase-contrast microscopy cell counts. H₂S and H₂ consumption rates were determined by pregrowing separate cultures on either H₂S or H₂ to acclimate the cells to a single energy source and then transferring 2×10^6 cells from late-log-phase cultures to 10-ml sealed serum bottles containing 5 ml of filter-sterilized, degassed spring water buffered at pH 3.0 with 5 mM citric acid and containing spring-relevant concentrations of H₂S [added as 30 μ M Na₂S(aq)] and H₂ (30 nM aqueous; provided by amending the headspace to 0.15% H₂ and allowing the bottles to equilibrate at 55°C in the laboratory with periodic shaking for 24 h). Upon inoculation, H₂ consumption was measured immediately for a time zero ($t = 0$) sample and then at 20-min intervals, and the samples were analyzed on a Shimadzu GC-8A electron capture detector with a stainless steel column (Shimadzu Scientific Instruments, Inc., Columbia, MD). Analyses of the levels of dissolved H₂S were performed as described above on 500- μ l samples taken at 15-min intervals. Killed controls were obtained by autoclaving cultures at 121°C for 45 min. The amounts of growth of the cultures with H₂S and H₂ were determined with the same culturing set up, except that headspace gases were replaced two to four times daily (depending on cell number) and Na₂S was added as a drip flow via a peristaltic pump.

In-field microelectrode assays. Electrochemical field analysis of H₂S concentrations was accomplished by using a portable DLK-60 potentiostat controlled by a laptop computer (Analytical Instrument Systems, Inc.) and micromanipulator. Voltammetry scans (1,000 mV/sec; initial holding potential of -0.1 V between -0.1 and -1.8 V [versus that for Ag/AgCl]) were acquired via a glass Au-amalgam solid-state working electrode, a platinum wire counter electrode, and a Ag/AgCl reference electrode constructed like that of Brendel and Luther (7). The H₂S signal assignment and calibrations were conducted by using spring water doped with freshly prepared standards (16). Vertical measurements of O₂ were carried out with a Clark-type microsensors and micromanipulator, using field methods, calibration techniques, and temperature compensation as described previously (32).

Scanning electron microscopy/energy-dispersive X-ray spectroscopy and sulfate analyses. Cultures were grown under the conditions described above, and the yellow precipitate that is common to these cultures was removed and sepa-

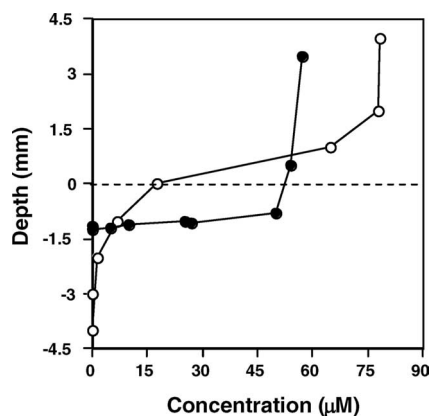


FIG. 2. Vertical H_2S and O_2 gradients in the microbial mats. Cyclic voltammetry was used to estimate H_2S concentrations in the center of the outflow channel approximately 100 cm downstream of the spring source, as shown by filled circles (●). The vertical profile of dissolved O_2 at a point 100 cm downstream of the spring source is shown by open circles (○). A dashed line represents the approximate upper boundary of the microbial mat.

rated from the culture media by centrifugation at $13,000 \times g$ for 5 min and then dried under vacuum conditions. The resulting material was applied to carbon tape on a sample stage and analyzed on a JEOL model 6100 scanning electron microscope (JEOL, Tokyo, Japan) equipped with an X-ray detector. The sample was subjected to 20 keV of incident energy, and measurements were made over a 50-s interval.

Production levels of sulfate by isolate 3684 were monitored in vitro by growing 10 ml of cells under standard conditions to $5 \cdot 10^5$ cells ml^{-1} and then concentrating the cells by centrifugation at $5,000 \times g$ for 5 min. Cells were resuspended in 1 ml of a synthetic medium [10 mM $(\text{NH}_4)_2\text{PO}_4$, 0.2 mM KH_2PO_4 , 2 mM NaCl, 1 mM MgCl, 0.5 mM CaCl_2 , 50 μM $(\text{NH}_4)_2\text{SO}_4$, 1.2 μM FeCl_3 , 0.5 ml trace element solution (2), 5 mM citric acid, pH 3.0] and used to inoculate 59 ml of the same medium. Na_2S was added to a final concentration of 200 μM , and 5.5-ml samples were removed at 12-h intervals and measured for H_2S utilization as described above, with 5.0 ml filtered through a 0.2- μm polytetrafluoroethylene filter and measured for SO_4 by using a Dionex ion chromatograph.

Nucleotide sequence accession numbers. The 16S rRNA gene sequences described in this study can be found as GenBank accession numbers EU545989 to EU545991 and the ITS sequences as accession numbers EU836320 to EU836324.

RESULTS

Spring chemistry. In a preceding study, sulfide constraints on the population distribution of an As(III) chemolithotroph were studied (10). Mapping H_2S gradients showed that measurable H_2S was primarily found in the yellow solid-phase zone (Fig. 1), which is a microbial mat comprised of filamentous microorganisms (primarily *Hydrogenobaculum* [19]) interwoven with a mineral phase made of up S^0 (21). At the point of discharge of the spring, $\text{H}_2\text{S}(\text{aq})$ was found to be 75 to 80 μM ; it declined to 5 to 10 μM at a distance of 3.5 m in the outflow channel at transect position 5 (Fig. 1) and was below detection at approximately 5 m (10). In the same study, O_2 gradients were also measured, and concentrations were found to be essentially opposite to those of H_2S ; i.e., $\text{O}_2(\text{aq})$ was undetectable in the first 100 cm in the outflow channel but then increased rapidly from there on.

The study described herein occurred approximately 18 months subsequent to that study, and H_2S levels were spot-checked to verify that the same gradient patterns were still

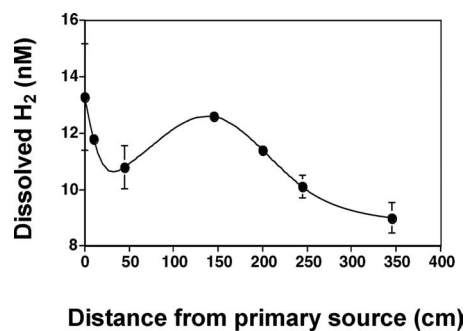


FIG. 3. Aqueous H_2 concentrations measured along the center of the outflow channel. The increase at 150 cm corresponds to input from a secondary spring source that contributes to the Dragon Spring outflow channel at approximately 140 cm. Data points indicate the means of three samples at each location. Error bars represent one standard deviation.

present (results not shown). In addition, to better understand potentially important geochemical gradients that may be of value for understanding in situ microbial metabolism and for designing ecologically relevant cultivation conditions, microelectrodes were used to examine H_2S and O_2 concentrations in the vertical dimension (Fig. 2). At a location approximately corresponding to transect site 3 (Fig. 1), H_2S concentrations in the water and within the upper 1 mm of mat appeared to remain nearly constant at $\sim 50 \mu\text{M}$ but then decreased sharply to the detection limit within ~ 2.5 mm (Fig. 2A). Vertical O_2 profiles were similarly steep, with O_2 levels being saturated 4 mm above the mat surface and rapidly changing to anaerobic conditions 2 to 3 mm inside the mat (Fig. 2B). Dissolved H_2 concentrations at the spring source were ~ 13 nM and decreased rapidly for approximately 50 cm. Aqueous H_2 then increased briefly, due to mixing with geothermal water from a second downstream source (Fig. 1), but again declined with distance (Fig. 3), presumably due to off-gassing and/or microbial consumption.

Ex situ assays. H_2S and H_2 consumption rates were studied to determine their relative importance as electron donors to the microorganisms inhabiting the S^0 deposition zone. Rates of H_2S loss (i.e., apparent consumption) in the sealed assay containers ranged from 35 to 50 $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g dry mat}^{-1}$, whereas H_2 consumption rates were approximately three orders of magnitude lower: 1 to 4 $\text{nmol} \cdot \text{min}^{-1} \cdot \text{g dry mat}^{-1}$ (Fig. 4; see also Fig. S1 in the supplemental material). In ex situ assays using heat-treated mat samples, the apparent H_2 and H_2S consumption was much lower or absent. For H_2S consumption results in heat-killed samples, incomplete heat killing cannot be ruled out, although much-slower abiotic oxidation likely also occurred due to the presence of oxygen unavoidably introduced during sample preparation at spring-side.

Phylogenetic analysis. A previous phylogenetic survey of the spring that included S^0 mat material from a position equivalent to site 5 (Fig. 1) found that *Hydrogenobaculum*-like signatures dominated the 16S rDNA clone libraries (19). To better understand the microbial community structure in the regions of the spring where H_2S and H_2 consumption were examined in the current study, additional 16S rRNA gene clones were am-

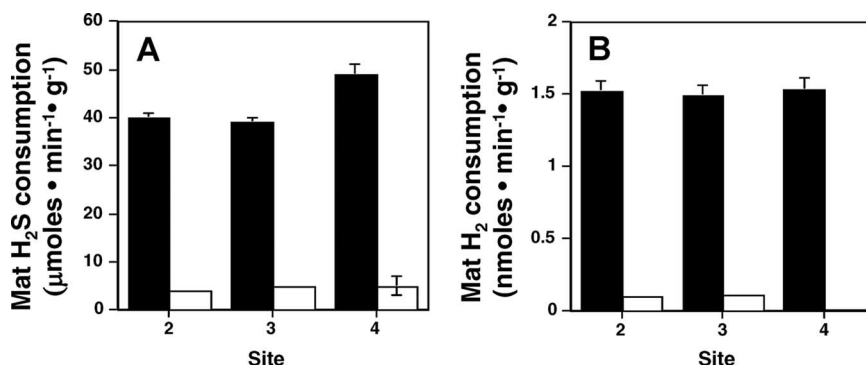


FIG. 4. Ex situ assays of apparent H₂S (A) and H₂ (B) consumption by whole-mat samples. Solid bars represent the activity of untreated mat material maintained at in situ temperatures. Open bars represent assays with heat-treated mat material. Site numbers refer to three discreet mat-sampling locations in the center of the outflow channel with respect to the distance from the spring source (2 = 35 cm, 3 = 115 cm, 4 = 225 cm). Each error bar represents one standard deviation of the mean from three replicate assays.

plified from mat DNA extracted from transect sites 2 and 4 within the central flow channel of the S⁰ deposition zone (Fig. 1). Of the 90 nearly full-length clones sequenced, 94% represented *Hydrogenobaculum*-like organisms (>99% identity to *Hydrogenobaculum acidophilum*, GenBank accession number D16296), falling into two distinct phylotypes with >99.9% nucleotide identity to one another; phylotype I comprised 71% of the *Hydrogenobaculum*-like clones, and the remainder were referred to as phylotype II.

Culturing and isolation. Filter-sterilized spring water was used as a basal medium in cultivation experiments aimed at isolating H₂S and H₂ chemolithoautotrophs from the mat exhibiting H₂S and H₂ consumption. Though the O₂ electrode experiments (Fig. 2B) indicated a continuum of aerobic, microaerobic, and anaerobic environments, only aerobic and microaerobic enrichments resulted in growth. Following several subcultures, clonal cultures were derived from positive enrichments by three rounds of dilution to a theoretical 0.5 cell · ml⁻¹. DGGE analysis was used to monitor isolation progress throughout; upon continued subculture, DGGE profiles were progressively less complex, reduced to a single band in each of the late-stage enrichment subcultures and remaining as such following dilution to extinction subcultures. A total of 30 isolates that were phylogenetically very similar to *Hydrogenobaculum* (>99% identity to *H. acidophilum*; GenBank accession number D16296) and that formed three distinct 16S phylotypes (see Fig. S2 in the supplemental material) were obtained. Isolate phylotypes I and II were identical to the above-mentioned phylotypes amplified from total mat DNA, whereas the 16S rRNA signature of phylotype III isolates was novel relative to amplicons derived from total mat DNA. Phylotypes I and III deviated from each other by 2 nucleotides, whereas phylotype II deviated from phylotypes I and III by 8 nucleotides over a 1,431-nucleotide region of the 16S gene.

Within each phylotype, cell morphology was invariable, and all isolates were identical with respect to 16S rRNA gene sequences and the cloned 350-bp ITS region. However, within each phylotype, the isolates displayed widely varying growth phenotypes. Some isolates would grow only on H₂S and some only on H₂, whereas others could grow with either energy source (Table 1). Further, some were found to grow best under microaerobic conditions, whereas others preferred fully aero-

bic conditions. Phylotype II contained isolates having all combinations of H₂S/H₂/O₂ utilization/requirement patterns. An aerobic isolate (strain YNP3684) capable of growth on either electron donor was maintained on sulfide for a period of 2 years, encompassing 32 culture transfers (1:50 dilution each) in the absence of H₂ (effective dilution = 10³²). Following this period of extended dilution and culturing, YNP3684 retained its ability to grow autotrophically on H₂, providing evidence that it was not a mixed culture.

Pure culture studies. Given that YNP3684 was phylogenetically identical to the dominant community sequence type and was capable of growth using either H₂S or H₂ as an electron donor and CO₂ as a carbon source, it was viewed to be ecologically relevant and ideal for modeling studies that would more closely examine H₂S or H₂ utilization under spring-relevant conditions. One set of experiments examined whether H₂S or H₂ exerted catabolite regulatory controls over the other and thus potentially influenced in situ utilization patterns. YNP3684 was first cultured separately with H₂S or H₂ (in filter-sterilized spring water) to acclimate the cells to a single energy source, and then late-log-phase cells were transferred to the same medium that now contained H₂S (30 μM) and H₂ (25 nM). In such experiments, consumption of both H₂S and H₂ was immediate and without a discernible lag (Fig. 5A). Initial consumption rates (first three time points) were 0.25 μmol · min⁻¹ · 10⁶ cells and 0.32 nmol · min⁻¹ · 10⁶ cells for H₂S and H₂, respectively. H₂S consumption appeared to re-

TABLE 1. Metabolic differences among *Hydrogenobaculum*-like isolates

16S phylotype	No. of isolates	No. of isolates capable of using indicated electron donor:			No. of microaerobic isolates ^a	No. of aerobic isolates ^a
		H ₂ S	H ₂	H ₂ S/H ₂		
I	9	3	2	4	0	9
II	18	15	0	3	6	12
III	3	2	0	1	0	3
Total	30	20	2	8	6	24

^a Isolates listed as microaerobic were incapable of growth with a headspace O₂ content of >2%, while aerobic isolates exhibited the best growth at a headspace O₂ content of 5.5% and are capable of growth with >10% O₂.

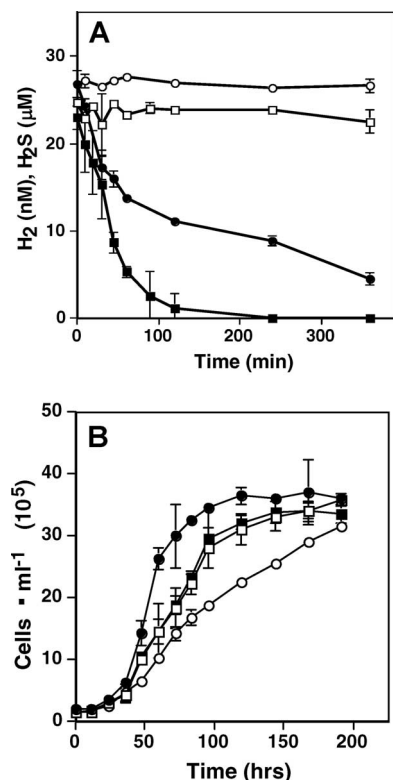


FIG. 5. H₂ and H₂S consumption and growth by *Hydrogenobaculum* strain 3684. (A) Time course consumption assay. Dissolved H₂ after the addition of autoclave-killed YNP3684 cells is shown by open circles (○), dissolved H₂S after the addition of autoclave-killed YNP3684 cells is shown by open squares (□), dissolved H₂ after inoculation with 2×10^6 cells ml⁻¹ is shown by filled circles (●), and dissolved H₂S following inoculation with 2×10^6 cells ml⁻¹ is shown by filled squares (■). (B) Growth of isolate YNP3684. H₂ (726 nM) is shown by filled circles (●), 30 nM H₂ + 60 μM H₂S is shown by filled squares (■), 60 μM H₂S is shown by open squares (□), and 30 nM H₂ is shown by open circles (○). Data are the means of triplicate samples. Error bars represent one standard deviation.

main nearly linear for 50 min (~ 5 μM), whereas H₂ consumption declined after 30 min ($0.03 \text{ nmol} \cdot \text{min}^{-1} \cdot 10^6 \text{ cells}$), when H₂(aq) decreased to <15 nM, a concentration roughly equal to that encountered in the spring source waters (Fig. 3).

Subsequent experiments then examined YNP3684 growth with H₂S and H₂ separately and in combination. As calculated at early time points (36 to 72 h) when cell concentrations were low and substrates were not limiting, the best growth rate (doubling time of $\sim 7.3 \pm 0.9$ h, $n = 3$) was observed with saturating levels of H₂ (726 nM) (Fig. 5B), although the overall-poorest growth rate (doubling time of $\sim 18.8 \pm 0.8$ h) occurred with 30 nM H₂, a concentration that still exceeded in situ levels in Dragon Spring by approximately a factor of 2. Growth rates with spring-relevant levels of H₂S were intermediate between these extremes (doubling time of $\sim 13.7 \pm 1.8$ h), and growth with H₂S and H₂ (doubling time of $\sim 12.9 \pm 0.9$ h) did not significantly improve growth statistically beyond that which occurred with H₂S alone (Fig. 5B).

Because background levels of S⁰ (solid phase) (Fig. 1) and SO₄ (1.2 mM) (21) in the spring prohibited experiments designed to track the fate of H₂S in the ex situ assays, experi-

ments were undertaken to determine the fate of H₂S in pure culture. Ion chromatography of culture fluids indicated that $\sim 80\%$ of added H₂S was converted to sulfate after 24 h of incubation with a cell culture at $1 \cdot 10^5$ cells ml⁻¹ (see Fig. 3A in the supplemental material). YNP3684 culture fluids were visibly yellow when grown on H₂S, whereas uninoculated media remained clear, suggesting that S⁰ may occur as an intermediate during enzymatic oxidation by this organism. Scanning electron microscopy/energy-dispersive X-ray spectroscopy analysis of solid phase culture constituents confirmed the presence of a sulfur-containing solid phase, consistent with the oxidation of H₂S to S⁰ (see Fig. 3B in the supplemental material).

DISCUSSION

Predictive studies concerning the energy sources that fuel primary production in geothermal environments have been very useful as first approximations. Molecular (16S rDNA) approaches estimate in situ metabolism based on physiologies inferred from phylogenetically closely related organisms that have been characterized (28, 33, 37), and thermodynamic predictions have been derived from free-energy yields calculated from the ion activities of electron donors and acceptors found in geothermal systems (4, 5, 18, 27, 33, 37). The present study had similar goals, while focusing on H₂ and H₂S; however, the experiments were designed to take a more-directed approach: i.e., quantifying presence, measuring utilization, and examining the characteristics of microorganisms capable of growth on these highly exergonic chemolithotrophic substrates.

A previous aqueous chemical analysis (10) agrees with the data obtained for this report (Fig. 2A), with both demonstrating significant levels of H₂S, and time course ex situ assays provide evidence of rapid microbe-based H₂S consumption (Fig. 4A). H₂S- and O₂-sensitive microelectrode experiments illustrated that H₂S penetration into the mat was limited, rapidly decreasing to below detection within the oxygenated region of the mat (Fig. 2), suggesting that H₂S consumption is linked to the presence of O₂. Similar experiments also demonstrated H₂ gradients (Fig. 3) and microbial consumption (Fig. 4B). The vertical and horizontal gradients of H₂S, H₂, and O₂ suggest a continuum of chemical energy gradients, providing numerous niche opportunities for populations having specialized or flexible metabolic needs. The latter was confirmed by the organisms obtained in the isolation exercises (Table 1) and, indeed, demonstrated optimum O₂ requirements exceeding that previously documented for *Hydrogenobaculum* (1, 12, 36).

The *Hydrogenobaculum* 16S rDNA signatures that dominated the community clone libraries were $\sim 99\%$ identical to those of *H. acidophilum*, which was characterized as being a microaerophile requiring H₂ and S⁰ for growth (36). Thus, physiologic inference would predict that organisms inhabiting the yellow S⁰ deposition zone would be engaged in H₂ oxidation and utilize the abundant S⁰ present in this region of the spring outflow channel (Fig. 1) (21). Results of the cultivation work, however, illustrate the potential problems associated with physiologic inference in general and, more specifically, the incorrect conclusions drawn if it is applied to the *Hydrogenobaculum*-like populations inhabiting this spring. Isolates

sharing identical 16S rRNA gene sequences (and, indeed, ITS sequences) differed in important ways with respect to the ecologically relevant electron donors they were capable of utilizing (Table 1). Such observations affirm those previously reported for organisms that are phylogenetically closely related (38) or, indeed, identical (20) but which exhibit widely varying physiologies.

The isolates obtained in this study were also important for other reasons. First, we drew attention to the observation that the overwhelming majority (90%) of the *Hydrogenobaculum* isolates were phylogenetically identical to the two major phylotypes (94% of all PCR clones) obtained from total community DNA. The use of filter-sterilized spring water as the basal growth medium along with O₂ concentrations predicted from the microelectrode work likely provided a more natural growth environment than synthetic media, which has historically tended to exclude the growth of ecologically relevant microorganisms (for an example, see reference 13).

Such isolates are also useful for pure-culture modeling studies, where results of ex situ experiments can be studied in more detail and under more-controlled conditions. When isolate YNP3684 (representing the dominant mat phylotype) was cultured in spring water with an average spring concentration of H₂S (30 μM) but H₂ levels (30 nM) considerably exceeding the maximum value observed at any location in the spring, H₂S consumption rates exceeded those of H₂ by three orders of magnitude, an observation completely consistent with the ex situ measurements (Fig. 4). Regardless of the substrate to which the cells had been preconditioned, utilization of both substrates was immediate and without a lag phase (Fig. 5A), suggesting constitutive expression of the enzymes involved. In some geothermal systems where microorganisms can be bathed in a continuous flux of multiple energy sources, constitutive utilization of several available energy sources may be the norm.

Another important observation derived from the pure-culture experiments speaks to the significance of enzyme kinetics when considering the relative importance of various energy sources in natural settings. Theoretical energy yields derived from calculating ΔG°_{rxn} fail to account for enzyme properties, the importance of which cannot be overstated. H₂ concentrations in the range of 5 to 10 nM have been shown to support microbial growth (24, 25, 37) and are similar to the concentrations observed in several Yellowstone geothermal features (Fig. 3) (37). However, these concentrations are considerably lower than the published K_m s derived from purified uptake hydrogenases (e.g., 0.92 μM H₂ for *Pyrodictium brockii* [30]; 19 μM for an *Anabaena* sp. [17]). The H₂ and H₂S consumption profiles exhibited by YNP3684 (Fig. 5A) suggest that over the range of concentrations of both substrates directly measured in the spring, utilization of H₂S surpassed that of H₂. Further, when spring-relevant concentrations of H₂ and H₂S were provided, consumption rates appeared closely related to actual growth, with this particular organism growing better with H₂S (Fig. 5B). Regular analysis of headspace gases in these experiments showed that the H₂-cultured cells never experienced H₂ concentrations lower than what were measured in the yellow S⁰ deposition zone (Fig. 3), indicating that the observed culture growth rates may exceed even that occurring in situ. Superior growth with H₂ was observed only with saturating concentra-

tions of H₂, levels that should be considered unrealistic given the concentrations measured in Dragon Spring and elsewhere in Yellowstone (29, 37).

While it is clear that the aerobic respiration of H₂ can provide substantial energy to chemolithotrophs, the potential energy gained from the oxidation of sulfide depends on the extent to which the sulfur is oxidized and may, in fact, be quite similar to that gained through H₂ oxidation under the spring conditions examined in this study (18). Several organisms, including *Thiobacillus thioparus* (23, 41) and *Desulfobulbus propionicus* (15), are capable of oxidizing H₂S to SO₄ under aerobic conditions. S⁰ production by this *Hydrogenobaculum* isolate (see Fig. S3B in the supplemental material) appears to be intermediate, as SO₄ production was also observed (see Fig. S3A in the supplemental material). While Rowe et al. (34) suggested that the presence of elemental sulfur was a major niche determinant for *Hydrogenobaculum*-like organisms they encountered in thermal tributaries feeding Lemonade Creek, our results indicate that the occurrence of S⁰ is due, at least in part, to the activity of the organisms present, with rates of abiotic H₂S oxidation apparently being a relatively minor contributor (Fig. 4).

In summary, this study directly examined the relative importance H₂S and H₂ as energy sources in support of primary productivity in a geothermal spring. Field experiments quantified both and measured consumption rates and were combined with cultivation and pure-culture assays to determine the dynamics of electron donor usage by an environmentally relevant isolate. The major conclusions drawn from this study are that phylogeny may not necessarily be a reliable predictor of physiology and that regardless of thermodynamic estimates, H₂S can dominate H₂ as an energy source in terms of availability, apparent in situ consumption rates, and growth-supporting energy.

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