CO$_2$ Uptake and Fixation by a Thermoacidophilic Microbial Community Attached to Precipitated Sulfur in a Geothermal Spring[▽][†]

Eric S. Boyd,[1][‡] William D. Leavitt,[2][§] and Gill G. Geesey[1]*

Department of Microbiology and Thermal Biology Institute, Montana State University, Bozeman, Montana 59717,[1] and School of Natural Science, Hampshire College, Amherst, Massachusetts 01002[2]

Carbon fixation at temperatures above 73°C, the upper limit for photosynthesis, is carried out by chemosynthetic thermophiles. Yellowstone National Park (YNP), Wyoming possesses many thermal features that, while too hot for photosynthesis, presumably support chemosynthetic-based carbon fixation. To our knowledge, in situ rates of chemosynthetic reactions at these high temperatures in YNP or other high-temperature terrestrial geothermal springs have not yet been reported. A microbial community attached to precipitated elemental sulfur (S° floc) at the source of Dragon Spring (73°C, pH 3.1) in Norris Geyser Basin, YNP, exhibited a maximum rate of CO$_2$ uptake of 21.3 ± 11.9 μg of C 10^7 cells^{-1} h^{-1}. When extrapolated over the estimated total quantity of S° floc at the spring's source, the S° floc-associated microbial community accounted for the uptake of 121 mg of C h^{-1} at this site. On a per-cell basis, the rate was higher than that calculated for a photosynthetic mat microbial community dominated by *Synechococcus* spp. in alkaline springs at comparable temperatures. A portion of the carbon taken up as CO$_2$ by the S° floc-associated biomass was recovered in the cellular nucleic acid pool, demonstrating that uptake was coupled to fixation. The most abundant sequences in a 16S rRNA clone library of the S° floc-associated community were related to chemolithoautotrophic *Hydrogenobaculum* strains previously isolated from springs in the Norris Geyser Basin. These microorganisms likely contributed to the uptake and fixation of CO$_2$ in this geothermal habitat.

The upper temperature limit for primary production via photosynthesis is ~73°C (7, 8, 11). At this temperature, photosynthesis is restricted to cyanobacteria of the genus *Synechococcus*, which generally inhabit alkaline environments (11). In acidic environments (pH < 4.0), the upper temperature limit for photosynthetic-based primary production is ~56°C. Under these conditions, phototrophic activity is restricted to the unicellular eukaryotic red algae *Cyanidium*, *Galdieria*, and *Cyanidioschyzon*, collectively referred to as "cyanidia" (6, 12, 31, 48). Primary production above this temperature in acidic environments occurs through chemoautotrophy, a metabolism restricted to prokaryotes.

Yellowstone National Park (YNP), WY, possesses numerous high-temperature (73 to 93°C) geothermal environments that are thought to support communities of microorganisms through chemoautotrophic-based primary production. Evidence for chemosynthesis in these environments is based on the recovery of 16S rRNA gene sequences that are affiliated with cultivated representatives of the phyla *Aquificae* and *Crenarchaeota*, many of which are capable of CO$_2$ fixation via the oxidation of hydrogen (H$_2$) and/or sulfide (HS$^-$) (15, 17, 21, 24, 26, 28, 41, 46). Surprisingly, CO$_2$ fixation has yet to be demonstrated in situ in YNP hot spring environments (acidic

or alkaline) where temperatures exceed the limits of photosynthesis and where primary production is thought to be driven by chemoautotrophic metabolism (14, 15, 28, 29).

Dragon Spring, an acid-sulfate-chloride (ASC) spring located in the Norris Geyser Basin of YNP, is a likely habitat for chemoautotrophic primary production. The pH of the water is ~3.1, and the temperature of the water at the source fluctuates from 65 to 78°C, which is well above the upper temperature limit for photosynthesis under acidic conditions. Potential electron donors for chemolithoautotrophic growth in the source water include hydrogen (H$_2$) and sulfide (S^{2-}) at concentrations of 13 nM and 65 μM, respectively (15). In addition, submerged substrata at the spring's source are blanketed by precipitates of elemental sulfur (S°), hereafter referred to as S° floc (23). Inventories of bacterial and archaeal 16S rRNA genes recovered from S° floc collected from the source of Dragon Spring indicate the presence of *Crenarchaeota* and *Aquificae* (4, 15). The latter are related to chemolithoautotrophic *Hydrogenobaculum* spp., representatives of which have recently been isolated from the spring (15). In the present study, we demonstrate uptake and fixation of CO$_2$ at a temperature of 73°C by a *Hydrogenobaculum*-dominated microbial community associated with S° floc collected from the source of Dragon Spring. This is the first direct evidence of CO$_2$ uptake in situ by a thermoacidophilic microbial community at a temperature that precludes photosynthesis in terrestrial geothermal springs.

* Corresponding author. Mailing address: Department of Microbiology and Thermal Biology Institute, Montana State University, P.O. Box 173520, Bozeman, MT 59717. Phone: (406) 994-3820. Fax: (406) 994-4926. E-mail: gill_g@erc.montana.edu.

‡ Present address: Department of Earth and Planetary Sciences, Harvard University, Cambridge, MA 02138.

§ Present address: Department of Chemistry and Biochemistry, Montana State University, Bozeman, MT 59717.

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MATERIALS AND METHODS

Collection and preparation of S° floc. Microorganisms that colonized S° floc in Dragon Spring (44°43′54.8″N, 110°42′39.9″W) served as the source of biomass for CO$_2$ uptake experiments. S° floc was collected from the spring source with a sterile 50-ml syringe and transferred to a sterile 30-ml serum bottle purged with sterile N$_2$ on the day of the experiment immediately prior to initiating the uptake
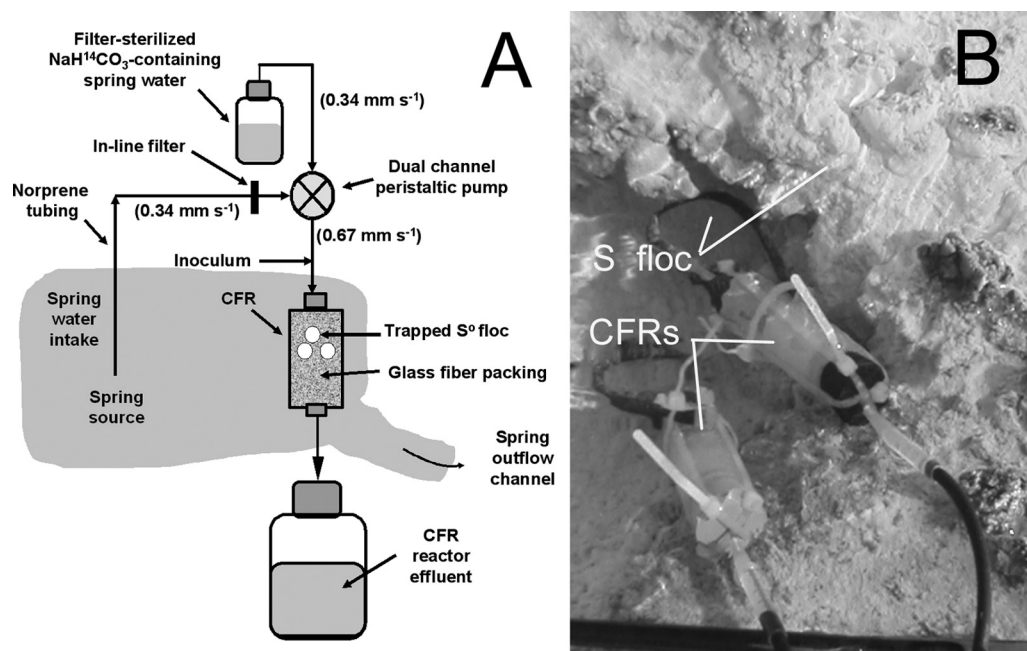
FIG. 1. Schematic (A) and image (B) of the CFR system used to measure CO_2 uptake by the S° floc-associated microbial community under conditions of continuous replenishment of fresh spring water.

reaction. The bottle was shaken to suspend and distribute the S° floc uniformly throughout the aqueous phase and used immediately as a source of microbial biomass for CO_2 uptake and fixation experiments as described below. Another portion of the S° floc suspension was frozen on dry ice for subsequent DNA- and RNA-based analyses. A portion of the frozen S° floc was later thawed and dried at 65°C for 48 h to determine volumetric solids content.

Static $^{14}CO_2$ uptake studies. Portions (10 ml) of spring source water and 0.25 ml of the S° floc suspension containing 1.0 mg (dry weight) of floc solids were injected by syringe and needle through the septa of presterilized, N_2-purged 12-ml serum bottles containing 0.1 ml of pH 3.0 citrate buffer (1 mM final concentration) unless otherwise indicated. An 18-gauge needle was used to introduce the S° floc suspension to serum bottles to minimize floc disintegration. Serum bottles were then submerged in the spring at its source and equilibrated to the spring water temperature (73°C). The $^{14}CO_2$ uptake reaction was initiated by syringe injection of NaH[^{14}C]O$_3$ (specific activity, 14.1 mCi/mmol; Sigma-Aldrich, St. Louis, MO). Killed controls were prepared by syringe injection of glutaraldehyde (0.5% final concentration) through the septa of serum bottles containing the same quantities of S° floc and spring water as live samples prior to the addition of NaH[^{14}C]O$_3$. Four serum bottles, three containing live samples and one serving as a killed control, were prepared for each experimental condition. All serum bottles were submerged in 73°C water at the source of Dragon Spring during the CO_2 uptake reaction. Unless otherwise noted, incubations were performed under ambient midday light. CO_2 uptake in the dark was determined in serum bottles wrapped in aluminum foil.

Electron donor and acceptor limitation on CO_2 uptake was investigated using the protocol described above with the following modifications. After 4 h of incubation of the S° floc-associated community in the presence of 7.7 μM NaH[^{14}C]O$_3$, the reaction mixture was supplemented with either O_2 (171 nM), H_2 (750 nM), or NaS$_2$ (65 μM) alone or H_2 (750 nM) or NaS$_2$ (65 μM) in combination with O_2 (171 nM) and then incubated for an additional hour before termination. The $^{14}CO_2$ uptake was compared to that of samples incubated for 5 h without electron donor/acceptor supplementation. The quantity of NaS$_2$ added to the reaction mixture (65 μM final concentration) did not affect the pH of the citrate-buffered reaction. The amount of NaS$_2$ added was intended to reflect the natural concentration of sulfide in the spring water (15). H_2 was added to achieve a saturating aqueous-phase concentration of 750 nM and thus is higher than that which exists in the spring source water (13 nM) but similar to that (726 nM) shown previously to promote the highest rate of growth of *Hydrogenobaculum* sp. strain YNP 3684, previously isolated from Dragon Spring (15). Since the concentration of O_2 in the source water is below the detection limit (13 μM) at this site and therefore not known (14), a quantity was added to

the reaction mixture (171 nM final concentration) well below this limit. Because the various experiments described above were conducted at different times over a period of several years, the date of sampling, the quantity of NaH[^{14}C]O$_3$ used in incubations, and the incubation time are presented with the results of each experiment.

Preliminary studies performed in August 2005 indicated that reduction of sample pH from 3.1 to 2.0 by syringe injection of concentrated HCl through the septa of the serum bottle to drive residual aqueous phase CO_2 into the headspace did not completely inhibit the biological CO_2 uptake reaction. Nold and Ward (39) reported that freezing stopped carbon fixation in cyanobacterial mat samples. Samples were therefore flash frozen on dry ice immediately after acidification to stop biological $^{14}CO_2$ uptake. Freezing may have resulted in some cell lysis, causing a portion of the fixed cell carbon to partition as dissolved organic carbon in the aqueous phase. This fraction of fixed carbon would likely have passed through the membrane during filtration and therefore would not have been included with the cell-associated fixed carbon. Since the extent of loss of fixed radiolabeled carbon was not determined, CO_2 uptake rates reported here may underestimate actual rates.

Upon return to the laboratory at Montana State University (MSU), each sample was thawed under a stream of N_2 to purge from the serum bottle any $^{14}CO_2$ not taken up by cells. The samples were immediately filtered through a polycarbonate membrane (0.22-μm pore size; Fisher Scientific, Pittsburg, PA), and the particulate material retained by the membrane rinsed with 10 ml of citrate-buffered water (pH 3.0) to reduce the amount of residual $^{14}CO_2$ associated with trapped particles and membrane surface that was not taken up by cells. The membranes were air dried and transferred to scintillation vials containing 10 ml of CytoScint scintillation cocktail (MP Biomedicals, Irvine, CA), and the radioactivity associated with the membranes was quantified by using a 1900CA Tri-Carb liquid scintillation counter (Packard, Downers Grove, IL).

$^{14}CO_2$ uptake under flowthrough conditions. Continuous-flow reactors (CFRs) were used to evaluate CO_2 uptake by the S° floc-associated microbial community during continuous replenishment of fresh spring source water on 14 September 2005 and repeated on 7 October 2006. The CFRs were constructed of rigid plastic cylinders (25-mm length, 9-mm radius) packed with 0.4 g of combusted glass wool fibers (550°C, 6 h) and capped with butyl rubber stoppers (Fig. 1). After assembly and submersion in the spring at the source, CFRs were perfused with 50 ml of spring source water using gas-impermeable Norprene tubing and a battery-powered peristaltic pump to equilibrate CFR contents to the surrounding spring source water temperature (73°C) and flow rate (0.67 mm s^{-1}). S° floc (1.0 mg [dry weight] of floc solids in 0.25 ml) prepared as described above for the static incubations was then injected with a syringe and 18-gauge

needle through the wall of the feed tubing at the CFR inlet while maintaining flow of source water through the reactor. The glass fiber packing served to retain the S° floc and associated microbial community in the CFR as water was pumped through the reactor.

A separate volume of water was transferred from the spring's source into a N$_2$-purged reservoir using gas-impermeable Norprene tubing containing an in-line filter (to remove suspended S° floc) and a battery-powered peristaltic pump. The water was supplemented with NaH[^{14}C]O$_3$ to achieve a final concentration of 7.7 μM (4 MBq liter^{-1}), and the pH was adjusted to 3.0. The water in the reservoir was then fed into the CFR after mixing the contents 1:1 (vol/vol) with the untreated water delivered directly from the spring's source by using a multichannel peristaltic pump in a "once through" continuous manner over a 2-h period. The mixture was pumped through the CFR at the same flow rate to 15.3 μM, while the concentration of unlabeled total dissolved inorganic carbon (DIC) in the spring water used for the uptake reaction was 4.4 mM (23, 29). Thus, the effluent of the CFR was collected in a waste reservoir and transported to MSU for disposal. A separate set of CFRs inoculated with the same quantity of S° floc but fed a mixture of NaH[^{14}C]O$_3$-supplemented spring water containing 0.5% (vol/vol) glutaraldehyde (final concentration) were included as killed controls.

CO$_2$ uptake by the S° floc-associated microbial community was terminated after the 2-h reaction period by flash freezing the CFRs and their contents on dry ice. Immediately prior to termination of the reaction, a 20-ml volume of CFR effluent from both live and killed experiments was collected in sterile Falcon tubes and frozen on dry ice for subsequent evaluation of efficiency of retention of S° floc-associated biomass by the reactors over the 2-h incubation. These samples were later thawed and filtered through 0.22-mm-pore-size polycarbonate membranes to collect the particulate phase. The membranes were rinsed with 1 mM citrate buffer (pH 3.0), air dried, and transferred to cocktail-containing scintillation vials, and the radioactivity was determined as described above. CFRs were thawed in the laboratory, and the glass fiber packing containing the S° floc-associated biomass was aseptically recovered and split into two 0.2-g subsamples. One subsample was dried and transferred to a scintillation vial containing scintillation cocktail. The radioactivity was measured as described above. The other 0.2-g subsample was used to characterize the glass fiber-associated microbial community as described below. The entire experiment was repeated, and the results (n = 2) were used to determine the statistical parameters as described below.

CO$_2$ uptake calculations. Biological CO$_2$ uptake rates were calculated by using the approach of Lizotte et al. (32) (see also the supplemental material). The approach is based on an increase in the rate of ^{14}CO$_2$ uptake with increasing concentration of ^{14}CO$_2$ when the added substrate represents an insignificant fraction of the total CO$_2$ present in the reaction mixture. The NaH[^{14}C]O$_3$ concentrations used in the uptake studies reported here ranged from 3.8 to 15.3 μM, while the concentration of unlabeled total dissolved inorganic carbon (DIC) in the spring water used for the uptake reaction was 4.4 mM (23, 29). Thus, the amount of radioactive NaH[^{14}C]O$_3$ added to the serum bottles represented an insignificant portion (≤0.35%) of the total CO$_2$ present.

The amount of ^{14}CO$_2$ taken up by the S° floc-associated community was based on the amount of radioactivity retained by the membrane filter after sample filtration (static incubations) or from the amount of radioactivity associated with the glass fiber matrix recovered from the CFRs after the radioactivity associated with killed controls was subtracted. The amount of ^{14}CO$_2$ taken up by the S° floc-associated community, the amount of NaH[^{14}C]O$_3$ present in the system, and the concentration of unlabeled DIC in the system were used to calculate total CO$_2$ uptake by the community (32). For experiments involving static incubations, the concentration of NaH[^{14}C]O$_3$ in the system was based on the amount added to initiate the reaction at t = 0. For experiments using CFRs, the concentration of NaH[^{14}C]O$_3$ in the system at any point in time during the 2-h incubation was calculated from the concentration of NaH[^{14}C]O$_3$ in the feed reservoir and the dilution factor due to mixing with water pumped directly from the spring's source before entering the reactors.

The rate of CO$_2$ uptake by the S° floc-associated microbial community was normalized to DNA as previously described (4). Briefly, DNA was extracted from 1.0 mg of S° floc, reacted with the DNA-binding fluorophore SYBR Gold I (Invitrogen, Carlsbad, CA), and quantified with a NanoDrop 3300 fluorimeter (Thermo Scientific, Wilmington, DE) using purified lambda phage DNA (Promega, Madison, WI) as a standard. This approach yielded 2.44 ± 0.53 μg of DNA g^{-1} (dry weight) of S° floc^{-1}. DNA-normalized CO$_2$ uptake rates were converted to uptake on a per-cell basis to facilitate comparison to rates reported previously for phototrophs. The conversion factor (7.0 fg of DNA cell^{-1}) was based on the size of the genome (3.2 Mb) of *Hydrogenobaculum* sp. strain 3684, a chemolithoautotroph belonging to the *Aquificaceae* within the *Aquificae* previously isolated from Dragon Spring (15). An average of 2.0 genome equivalents per cell for thermophilic microorganisms at stationary phase growth (1, 33, 35)

and an average molecular weight of double-stranded DNA of 660 Da (45) were also used to determine the conversion factor. Exponential-phase cultures of thermophiles can contain a greater number of genome equivalents per cell (≥4) than stationary-phase cultures (35). Thus, the conversion factor used in the present study may result in an underestimate of the actual CO$_2$ uptake rate per cell. Details of CO$_2$ uptake calculations are presented in the supplemental material.

Statistical analyses. The standard deviation among replicates was calculated according to the formula: SD = $\sqrt{(A^2 + B^2)}$, where A is the average standard deviation of replicate CO$_2$ uptake measurements (n = 3 for static experiments; n = 2 for CFR experiments, unless otherwise stated) and B is the standard deviation of replicate DNA determinations on different subsamples of S° floc (n = 3 for static or CFR experiments). P values were obtained by using a two-tailed Student t test. Differences in CO$_2$ uptake under the various conditions tested were considered significant if P values were ≤0.05.

Incorporation of inorganic carbon into cellular organic material. Incorporation of radiolabeled substrate into cellular carbon pools followed the approach of Brock and Brock (10). S° floc suspension (5 ml) and fresh unfiltered spring source water (45 ml) were added by syringe and needle to six sterile N$_2$-purged 150-ml serum bottles sealed with serum stoppers and equilibrated in the source water of Dragon Spring. After equilibration, 5 ml of air (171 nM final dissolved O$_2$ concentration) and 100 μl of NaH[^{14}C]O$_3$ (16.4 MBq ml^{-1}, 2.11 GBq mmol^{-1}) were introduced with syringe and needle to the serum bottles. Three serum bottles were immediately chilled on ice to inhibit biological carbon fixation and serve as killed controls. The remaining serum bottles were returned to the spring and incubated for 4 h at spring source water temperature (70°C) under ambient light during the peak photoperiod on 12 November 2008. After incubation, the serum bottles were chilled on ice to stop biological carbon fixation. All serum bottles were stored on ice during transport to the laboratory at MSU. Upon return to the laboratory, the contents of each serum bottle were mixed, evenly distributed into three centrifuge tubes, and centrifuged at 5,000 × g for 10 min. The supernatant was decanted and replaced with either 2 ml of cold 0.5 N HClO$_4$ to extract the low-molecular-weight pool, 2 ml of hot 0.5 N HClO$_4$ to extract nucleic acids and acid-labile peptides, or 2 ml of hot 1 N NaOH to extract protein. The tubes containing hot HClO$_4$ or NaOH were boiled (~93°C) for 20 min, while the tubes containing cold HClO$_4$ were chilled (0 to 1°C) on ice for 20 min. The tubes were then centrifuged at 5,000 × g for 10 min, and the supernatant was decanted into scintillation vials containing scintillation cocktail. The radioactivity was counted in a scintillation counter as described above. The amount of ^{14}CO$_2$ incorporated into the various cellular pools was calculated after the subtraction of uptake associated with killed controls.

RT-PCR. Triplicate 2-mg quantities of S° floc (500 μl) used to inoculate serum bottles and CFRs and triplicate 50-mg quantities of glass fiber packing material recovered from the glutaraldehyde-free CFRs after the 2-h incubation were subjected to bead beating-sodium dodecyl sulfate RNA extraction as previously described (2). The replicate extracts for each set of subsamples were pooled and subjected to reverse transcription PCR (RT-PCR) using an Access RT-PCR kit (Promega) as previously described (4). Primers used in RT-PCR (1070F [bacterial] or 931F [archaeal] and 1492R-GC) targeted the community 16S rRNA. Negative control reactions were performed in the absence of reverse transcriptase.

DGGE. The 16S cDNA generated by RT-PCR was evaluated by denaturing gradient gel electrophoresis (DGGE) using a 7% polyacrylamide gel containing a vertical gradient of 50 to 70% denaturant (bacterial 16S cDNA amplicons) or 35 to 55% denaturant (archaeal 16S cDNA amplicons) (4).

Cloning, sequencing, and analysis of cDNA. Cloning, sequencing, and analysis of bacterial 16S cDNA amplicons was performed as previously described (3). The single DGGE band obtained from the archaeal amplicons was excised, purified, and sequenced as previously described (20). CLUSTAL X (version 2.0.8) (30) was used to align sequences and to create distance matrices for use in identifying and clustering operational taxonomic units (OTU) using DOTUR (42). DOTUR calculations used the furthest-neighbor algorithm and a precision of 0.01. A representative of each OTU was subjected to BLASTn to determine the phylogeny of the closest affiliated sequence type.

Nucleotide sequence accession numbers. All 16S cDNA sequences have been deposited in the GenBank, DDBJ, and EMBL databases under the accession numbers DQ3984487 to DQ398805.

RESULTS

The results from a series of experiments performed on 15 July 2006 indicated that glutaraldehyde treatment inhibited
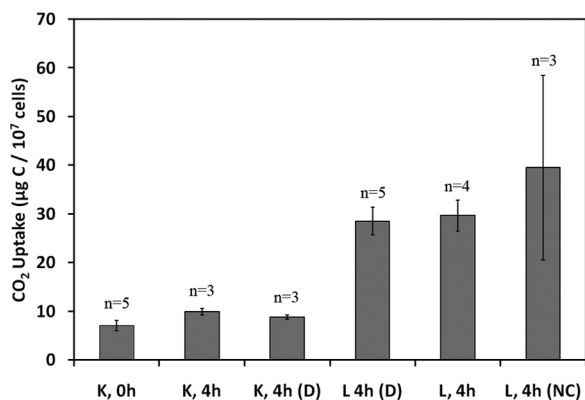
FIG. 2. Uptake of $^{14}CO_2$ by a S° floc-associated microbial community under static conditions in the presence of 15.3 μM NaH[^{14}C]O_3 after no incubation (0 h) or after 4 h (4 h) of incubation. All incubations were performed in the light with the exception of those labeled D, denoting incubations performed in the dark. Live (L) and glutaraldehyde-killed control (K) reactions are indicated. All incubations contained 1 mM citrate buffer (pH 3.0) unless otherwise indicated (NC), which contained no citrate. n, Number of biological replicates.

uptake of CO_2 by the S° floc-associated microbial community in pH 3.0 Dragon Spring source water at 73°C. The quantity of radiolabeled substrate taken up by the community after 4 h of incubation in glutaraldehyde-treated source water containing 15.3 μM NaH[^{14}C]O_3 was not significantly different from that taken up after no incubation upon exposure to source water containing the same supplements (Fig. 2). This was the case regardless of whether the incubations were performed in the light or the dark ($P = 0.11$ and 0.08, respectively). The amount of radioactivity recovered from samples of spring water without added S° floc incubated for 4 h in the presence of 15.3 μM NaH[^{14}C]O_3 was not significantly different ($P = 0.43$) from that recovered from glutaraldehyde-killed S° floc-containing source water incubated in the presence of the same quantity of NaH[^{14}C]O_3 for the same period of time (data not shown). This result suggests that the residual radioactivity associated with the glutaraldehyde-treated S° floc-containing samples, hereafter referred to as killed controls, was NaH[^{14}C]O_3 that was bound to the membrane or associated with material in the source water other than the added floc trapped by the membrane.

The quantity of radiolabeled substrate taken up by the S° floc-associated microbial community incubated in glutaraldehyde-free source water containing 15.3 μM NaH[^{14}C]O_3 under mid-day ambient light was significantly greater ($P = 0.01$) than that taken up by the floc-associated community in glutaraldehyde-treated source water incubated under otherwise similar conditions (Fig. 2). The difference represents uptake of CO_2 by the S° floc-associated microbial community. Other microbial populations present in the source water did not appear to contribute significantly to CO_2 uptake under these conditions. The amount of radioactivity recovered from NaH[^{14}C]O_3-supplemented source water incubated for 4 h without added S° floc was not significantly different ($P = 0.28$) from that recovered from source water receiving neither NaH[^{14}C]O_3 or S° floc and incubated for the same period of time (data not shown).

Although photosynthesis is not thought to occur at the combined low pH and high temperature of Dragon Spring source water (5, 6, 12), a dark incubation was performed to compare CO_2 uptake under this condition to that reported above in the light. CO_2 uptake in the dark was not significantly different ($P = 0.89$) from that in the light during peak photoperiod under otherwise similar conditions (15.3 μM NaH[^{14}C]O_3, 4-h incubation) (Fig. 2). This indicates that essentially all CO_2 uptake by the S° floc-associated microbial community at 73°C in the presence of light can be attributed to the activities of chemolithoautotrophs that do not depend on photosynthesis. In view of these results, all of the studies reported below were conducted under ambient light.

CO_2 uptake by the S° floc-associated community in the studies described above was determined in the presence of citrate buffer to maintain the reaction mixture at the source water pH (3.0). An additional incubation was performed on this date to determine the effect of citrate on CO_2 uptake by comparing $^{14}CO_2$ uptake in the presence or absence of the buffer. The presence of buffer had no significant effect ($P = 0.50$) on $^{14}CO_2$ uptake other than to reduce variability among replicates (Fig. 2).

A portion of the CO_2 taken up by the S° floc-associated microbial community was fixed into the cell biomass. Significantly more radioactivity (introduced as NaH[^{14}C]O_3) was recovered in the hot $HClO_4$ extract (nucleic acid and acid-labile peptide cellular pool) of samples incubated for 4 h at 70°C than from extracts of NaH[^{14}C]O_3-amended samples that were immediately chilled on ice ($P = 0.006$). The total amount of CO_2 incorporated into this pool was 0.38 μg of C g (dry weight) of S° floc^{-1} based on the previously determined spring water DIC concentration of 4.4 mM (23, 29). The quantities of radioactivity associated with the low-molecular-weight (cold $HClO_4$) and protein (NaOH) pools of incubated samples were not significantly different from those of the killed controls (data not shown).

The rate of $^{14}CO_2$ uptake in live samples over a 2-h period increased linearly with NaH[^{14}C]O_3 concentration (3.8 to 15.3 μM) for different amounts (0.5 and 1.0 mg) of added S° floc in an experiment performed on 2 August 2005 (Fig. 3A). These results qualify the data for use in calculating the rate of uptake of unlabeled CO_2 in the samples by the approach of Lizotte et al. (32). In the presence of 1.0 mg of S° floc and 7.7 μM NaH[^{14}C]O_3, detectable $^{14}CO_2$ uptake occurred for up to 3 h, but the rate decreased over this period of time (Fig. 3B). A maximum rate of $^{14}CO_2$ uptake (21.3 ± 11.9 μg of C 10^7 cells^{-1} h^{-1}) was observed during the first hour of incubation, decreasing to 18.1 ± 9.7 μg of C 10^7 cells^{-1} h^{-1} when averaged over a 2-h incubation period. These results indicate that the reaction was limited shortly after initiation.

Electron donor or acceptor limitation of CO_2 uptake under the same conditions described above (7.7 μM NaH[^{14}C]O_3) was evaluated through supplementation experiments on 20 August 2005. Supplementation of O_2 (171 nM) alone significantly ($P = 0.016$) stimulated CO_2 uptake relative to the unsupplemented control, suggesting that the reaction was limited by this electron acceptor (Fig. 4). Supplementation of NaS_2 (65 μM) alone or in combination with O_2 (171 nM) did not significantly stimulate ($P = 0.37$ and $P = 0.10$, respectively) CO_2 uptake (Fig. 4). H_2 supplementation (750 nM) appeared to significantly inhibit ($P = 0.05$) CO_2 uptake relative to un-
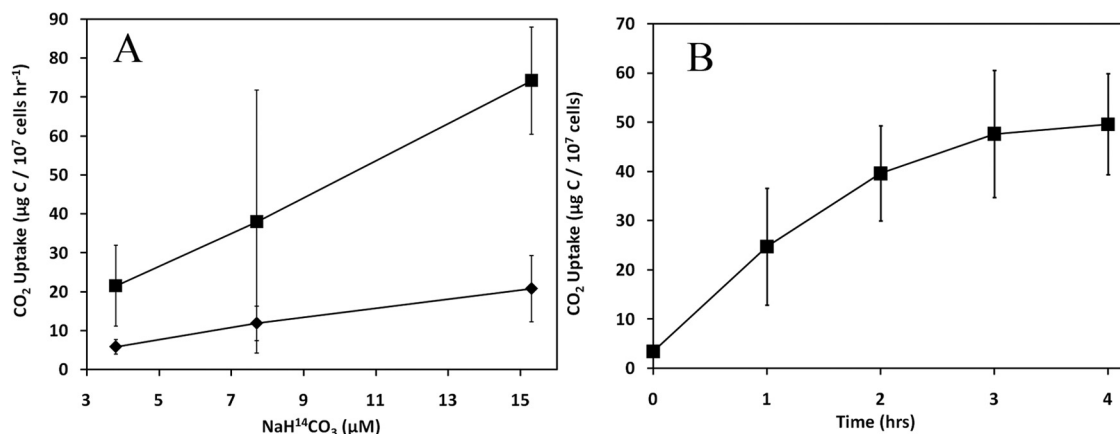
FIG. 3. CO$_2$ uptake under static conditions by the microbial community associated with 0.5 mg (◆) or 1.0 mg (■) of S° floc after 2 h of incubation in Dragon Spring source water supplemented with a range of NaH[^{14}C]O$_3$ concentrations (A) or associated with 1.0 mg of S° floc over a 4-h incubation in Dragon Spring source water supplemented with 7.7 μM NaH[^{14}C]O$_3$ (B). Means and standard deviations are based on results from experiments performed in triplicate ($n = 3$).

supplemented controls (Fig. 4). Furthermore, H$_2$ inhibition was not alleviated by the addition of O$_2$ (Fig. 4).

In view of the evidence of O$_2$ limitation of CO$_2$ uptake by the S° floc-associated community in samples incubated under static conditions, the reaction was subsequently carried out in a CFR containing 1.0 mg of S° floc exposed to 7.7 μM NaH[^{14}C]O$_3$ in the presence of a fresh supply of flowing spring source water. A total CO$_2$ uptake rate of 13.5 ± 0.9 μg of C 10^7 cells^{-1} h^{-1} was obtained when averaged over a 2-h incubation period. The uptake rate under these conditions was not significantly different ($P = 0.23$) from the rate (18.1 ± 9.7 μg of C 10^7 cells^{-1} h^{-1}) averaged over the same 2-h incubation period in static incubations containing the same quantity of S° floc and concentration of radiolabeled substrate. Comparison of the volume and radioactivity of solution in the feed reservoir to
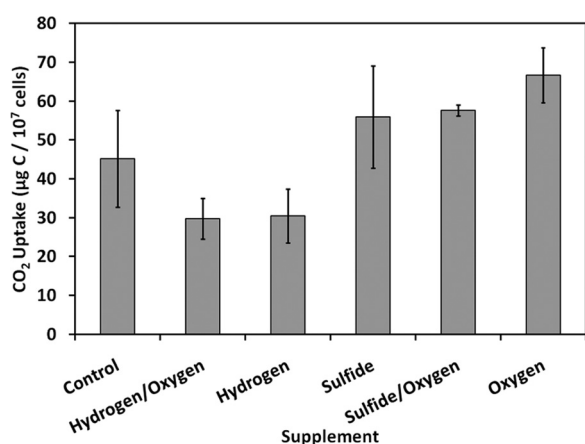
that of the CFR effluent indicated that the concentration of radiolabeled substrate in the CFRs did not differ by more than 7% over the 2-h incubation period (data not shown). Furthermore, the amount of radioactivity associated with the particulate phase of the effluent from the live and killed CFRs was not significantly different. This result indicates that the inoculum (S° floc-associated biomass) introduced at the beginning of the incubation that participated in the uptake of radiolabeled substrate was retained in the CFRs over the duration of the experiment (data not shown).

The composition of the microbial community associated with the glass fiber matrix in the CFRs at the end of the 2-h incubation was compared to that associated with the S° floc used to inoculate the reactors. DGGE analysis of bacterial 16S cDNA from RNA recovered from the S° floc used as CFR inoculums and from the CFR glass fiber matrix yielded 14 and 12 bands (phylotypes), respectively. All 12 bands from the CFR glass fiber matrix were present in the S° floc inoculum. All 12 DGGE phylotypes that were shared between communities migrated to a similar position in a 50 to 70% denaturing gel (data not shown), suggesting that these 16S cDNA sequences have similar mol% G+C content. Similarly, DGGE analysis of archaeal 16S rRNA indicated a single, shared phylotype in both the inoculum and the material recovered from the CFR glass fiber matrix. The results suggest that the microbial community did not change significantly during the uptake reaction as fresh filtered spring water was continuously pumped through the reactors. The results also indicate that the S° floc-associated microbial community used for the CO$_2$ uptake reaction in the static incubation studies was not significantly different from that responsible for the reaction in the CFRs.

A clone library was constructed from bacterial 16S cDNA derived from RNA extracted from the S° floc used as the CFR inoculum. At a 100% sequence identify threshold, a total of 14 OTU were present, 6 of which contained multiple clones and 8 of which represented singlets (Table 1). Analysis of representatives from each OTU indicated that 12 of the 14 OTUs exhibited high affiliation (>98% sequence identities) to chemolithoautotrophic *Hydrogenobaculum* sequences previously isolated

FIG. 4. CO$_2$ uptake by microbial community associated with 1.0 mg of S° floc after a 5-h incubation under static conditions in Dragon Spring source water supplemented with 7.7 μM NaH[^{14}C]O$_3$ after addition in the final hour of either 750 nM hydrogen (hydrogen), 65 μM sulfide (sulfide), 191 nM O$_2$ (oxygen), 750 nM hydrogen plus 191 nM O$_2$ (hydrogen/oxygen), 65 μM sulfide plus 191 nM O$_2$ (sulfide/oxygen), or no electron donor or acceptor (control). Means and standard deviations are based on results from experiments performed in triplicate ($n = 3$).

TABLE 1. Diversity of RT-PCR-amplified bacterial and archaeal 16S cDNA recovered from sulfur flocs

OTU[a]	Frequency[b]	Closest affiliated organism[c]	Phylum	% Identity[d]
B1	18	*Hydrogenobaculum* sp. strain NOR3L3B (ZAJ320225)	*Aquificae*	99.8
B2	4	*Hydrogenobaculum* sp. strain Y04ANC1 (AM259505)	*Aquificae*	100.0
B3	7	*Hydrogenobaculum* sp. strain Y04ANC1 (AM259505)	*Aquificae*	99.8
B4	1	*Hydrogenobaculum* sp. strain Y04AAS1 (AM259504)	*Aquificae*	99.3
B5	5	*Hydrogenobaculum* sp. strain Y04AAS1 (AM259504)	*Aquificae*	100.0
B6	1	*Hydrogenobaculum* sp. strain Y04AAS1 (AM259504)	*Aquificae*	99.5
B7	2	*Hydrogenobaculum* sp. strain NOR3L3B (ZAJ320225)	*Aquificae*	99.8
B8	2	*Hydrogenobaculum* sp. strain Y04ANC1 (AM259505)	*Aquificae*	99.8
B9	1	*Hydrogenobaculum* sp. strain NOR3L3B (ZAJ320225)	*Aquificae*	99.1
B10	1	*Hydrogenobaculum* sp. strain Y04ANC1 (AM259505)	*Aquificae*	98.8
B11	1	*Hydrogenobaculum acidophilum* H55 (AY268103)	*Aquificae*	99.0
B12	1	*Hydrogenobaculum acidophilum* H55 (AY268103)	*Aquificae*	99.2
B13	1	*Cupriavidus* sp. strain JB1B4 (EU375663)	*Betaproteobacteria*	86.7
B14	1	*Ralstonia taiwanensis* LMG 19424 (AF300324)	*Betaproteobacteria*	100.0
A1	NA	*Thermocladium modestius* (AB005296)	*Themoprotei*	97.6

[a] OTU were as defined by DOTUR using a sequence identity threshold of 100%. B, bacteria; A, archaea.
[b] That is, the OTU frequency in the bacterial 16S cDNA library. NA, not applicable.
[c] That is, the cultured organism with the closest affiliated 16S rRNA gene sequence. The accession number is given in parentheses.
[d] That is, the percent identity with the closest affiliated sequence.

from the Norris Geyser Basin belonging to the *Aquificaceae* within the *Aquificae* (15–17, 19). A smaller fraction of the OTUs were affiliated with *Cupriavidus* sp. strain JB1B4 and *Ralstonia taiwanensis* LMG 19424, both of which are members of the *Betaproteobacteria* (Table 1). The sole archaeal 16S rRNA sequence (as assessed by DGGE) was affiliated (97.6%) with *Thermocladium modestius*, a crenarchaeon isolated from a geothermal area in Japan (24).

DISCUSSION

The results confirm earlier predictions that carbon fixation occurs at temperatures above the maximum temperature for photosynthesis in habitats of YNP containing alternative sources of energy that support chemosynthesis. If phototrophs had contributed to CO_2 uptake by the S° floc-associated microbial community at 73°C, one would predict the rate of uptake in the light to be greater than that in the dark. In habitats with comparable temperatures but higher pH where photosynthetic prokaryotes of the genus *Synechococcus* outcompete other life forms, dark CO_2 uptake typically represents less than 10% of the total CO_2 taken up (8, 9, 38). The insignificant difference in uptake rates in the light and dark and evidence of incorporation of radiolabeled inorganic carbon into cellular DNA indicates that fixation of carbon is coupled to S° floc-associated chemosynthesis rather than photosynthesis at the source of Dragon Spring. To our knowledge, this is the first direct evidence of in situ CO_2 uptake and fixation by a thermoacidophilic microbial community at a temperature above that which supports photosynthesis.

The limited size bacterial 16S cDNA library constructed from S° floc-associated RNA was dominated (92% of total clones) by sequences exhibiting close affiliation (>98% identity) to *Hydrogenobaculum* spp. The 12 OTU present in the bacterial 16S rRNA clone library that are related to *Hydrogenobaculum* spp. are genetically similar (average sequence divergence of 0.0087%) and thus may correspond to the 12 shared phylotypes that migrated to nearly the same position in

the DGGE acrylamide gel. Members of this genus are acidophilic chemolithoautotrophs that generally use H_2, S°, $S_2O_3^-$ or HS^- as electron donor and O_2 or oxidized sulfur compounds such as S° or thiosulfate as electron acceptor to fix CO_2 (17, 41, 43, 44, 47). In Dragon Spring, as well as other acidic springs, representatives of this genus have been proposed to obtain their cell carbon through CO_2 fixation (22, 23, 25, 34, 46). They have also been shown to physically attach to sulfur (36). The populations of *Hydrogenobaculum* spp. detected in the clone library may therefore contribute to the chemoautotrophic CO_2 uptake and fixation by the S° floc-associated microbial community at the source of Dragon Spring.

Thermophilic chemolithoautotrophs isolated from high-temperature environments have been shown to use O_2, NO_3^-, Fe^{3+}, and various oxidized sulfur species as the terminal electron acceptor (14–17, 27, 47). Past studies indicate that the source water of Dragon Spring contains 17 μM NO_3^- and an O_2 concentration that is below detection (13 μM) (14, 15, 23, 29). The stimulation in the rate of CO_2 uptake by the S° floc-associated microbial community under static conditions upon addition of O_2 indicates that uptake under these conditions was O_2 limited. Dissolved O_2 is maintained at undetectable concentrations at the source of Dragon Spring through a reaction with HS^- in the source water to form polysulfide (S_n) and $S_2O_3^-$, which subsequently disproportionate to S° and HS^- (40). *Hydrogenobaculum acidophilum* (formerly *Hydrogenobacter acidophilis*), one of the few strains of this genus for which the oxygen requirements have been determined, exhibits optimum growth at partial pressures (pO_2) between 2 to 10%, well above the ambient concentration in Dragon Spring source water (43). The threshold concentration of O_2 for carbon fixation in populations of the S° floc-associated community may therefore be lower than that of the few isolates examined to date. Supporting this hypothesis is evidence that As(III) oxidation by *Hydrogenobaculum* sp. strain H55 isolated from Dragon Spring was only detected under microaerophilic (~1% O_2) conditions (16). The S° floc-associated microbial populations may also be capable of coupling carbon fixation to alter-

native electron acceptors present in the source water such as nitrate. However, their involvement in chemoautotrophic carbon fixation under conditions of O$_2$ limitation is beyond the scope of the present study.

Thermophilic chemolithoautotrophs isolated from high-temperature environments have been shown to use H$_2$ and various reduced sulfur species as electron donors (energy source) (14–17, 27, 47). Past studies indicate that the source water of Dragon Spring contains 13 nM H$_2$ and 60 to 80 μM H$_2$S (14, 15, 23, 29). CO$_2$ uptake by the S^o floc-associated community was inhibited by high concentrations of H$_2$. Whereas H$_2$ has been shown to inhibit heterotrophic metabolism in thermophiles (13, 18, 49, 50), this is, to our knowledge, the first report of H$_2$ inhibiting a reaction associated with chemoautotrophic metabolism at a high temperature. The concentration of H$_2$ used to evaluate H$_2$ limitation (750 nM), while over an order of magnitude greater than the ambient concentration (13 nM) in the source water of the spring, was similar to that (726 nM) shown previously to promote the highest rate of growth of isolate YNP 3684, a chemolithoautotrophic *Hydrogenobaculum* sp. isolated from Dragon Spring (15). The inhibitory effect of 750 nM H$_2$ on CO$_2$ uptake in the present study suggests that the response of this isolate to H$_2$ is not representative of that of the S^o floc-associated community. Further investigation of the effect of H$_2$ on chemosynthetic processes at the source of Dragon Spring is clearly warranted but is beyond the scope of the present study.

Sequences related to the crenarchaeon *T. modestius*, the only archaea detected in the S^o floc-associated community by DGGE analysis of 16S cDNA, have previously been recovered from Succession Spring, a nearby thermal feature in the Norris Geyser Basin that has water chemistry similar to that of Dragon Spring (23, 35). *T. modestius* does not grow autotrophically using H$_2$ and CO$_2$, although heterotrophic growth under anaerobic conditions at 75°C over the pH range of 4.0 to 4.5 is stimulated by addition of CO$_2$ to the culture headspace (24). Although the limited community analysis reported here does not indicate involvement of archaea in CO$_2$ uptake by the S^o floc-associated microbial community, deeper sampling of 16S rRNA gene sequences with more diverse primer sets or 454 V6 tag sequencing may reveal autotrophic archaeal populations associated with the microbial community at this site.

Rates of CO$_2$ uptake normalized to chlorophyll (Chl) content have been reported for photosynthetic organisms at temperatures comparable to that used here to investigate chemosynthetic CO$_2$ uptake (8, 9, 38). The photosynthetic efficiency and Chl a content of cells vary with the temperature and radiation level in *Synechococcus* (7, 38), the only known phototroph capable of growth at temperatures approaching that (73°C) used for incubations in the present study. Brock and Brock (9) reported a cellular Chl a content of 81 fg of Chl a cell^{-1} for *Synechococcus* spp. taking up ^{14}CO$_2$ at 71°C at a rate of 1,533 cpm μg^{-1} Chl a h^{-1}. Based on these values and a previously reported spring water total DIC concentration of 55 μg ml^{-1} (8), a total CO$_2$ uptake rate of 0.47 μg of C 10^7 cells^{-1} h^{-1} was obtained when cpm was converted to dpm using the counting efficiency (65.5%) determined for the present study. A higher total CO$_2$ uptake rate (1.9 μg of C 10^7 cells^{-1} h^{-1}) was calculated from the ^{14}CO$_2$ uptake rate (2.3 × 10^8 cpm 186 mg of Chl a^{-1} m^{-1} h^{-1}) measured in an earlier study at the

same site (8). More recently, Miller et al. (38) reported a ^{14}CO$_2$ uptake rate of 2,304 cpm μg of Chl a^{-1} h^{-1} for dispersed cells of a *Synechococcus* mat from another alkaline spring incubated at 70°C in water containing 67.5 μg of DIC ml^{-1}. This reflects a total CO$_2$ uptake rate of 1.1 μg of C 10^7 cells^{-1} h^{-1} based on the experimentally determined value of 64 fg of Chl a cell^{-1} from a 45°C exponential culture of a *Synechococcus* sp. isolated from the mat (37). The rates calculated above for the alkalinophilic phototrophic *Synechococcus* communities are at least an order of magnitude less than the 18.1 and 13.5 μg of C 10^7 cells^{-1} h^{-1} calculated for the S^o floc-associated microbial community over 2-h incubations under static and flowing conditions, respectively. The higher CO$_2$ uptake rates by the S^o floc-associated microbial community suggests that chemoautotrophic acidophiles are better adapted than photoautotrophic alkalinophiles to take up CO$_2$ at the upper temperature limit for photosynthesis.

S^o floc forms an ~0.5-cm-thick blanket over an area of ~300 cm by 50 cm at the spring's source (Fig. 1B). Extrapolation of the maximum CO$_2$ uptake rate exhibited by the microbial community associated with 0.25 ml of S^o floc in the static incubation experiments to the total volume of S^o floc at the source yielded a total CO$_2$ uptake rate of 121 mg of C h^{-1}. Additional studies are needed to determine the biomass production resulting from this rate of CO$_2$ uptake before the significance of chemosynthesis under these conditions can be established.

In summary, a microbial community associated with S^o floc at the source of Dragon Spring, YNP, coupled the uptake and fixation of inorganic carbon into cellular organic matter using light-independent chemosynthetic metabolism at a temperature and pH that do not support photosynthesis. Uptake rates measured in situ at 73°C and pH 3.1 were at least an order of magnitude higher than those reported for photosynthetic *Synechococcus* microbial mats in alkaline springs at slightly lower temperatures coinciding with the upper limits for photosynthesis. Chemolithoautotrophic *Hydrogenobaculum* spp. appeared to be important members of the S^o floc-associated microbial community and likely contributed to the CO$_2$ uptake rates measured under the conditions of the present study.

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