Use of stable isotope probing to identify bacterial species involved in CO$_2$ fixation in the water just below the oxycline of the meromictic Lake Suigetsu, Japan

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Abstract: In this study, stable isotope probing was used to identify a bacterial community involved with bicarbonate fixation in the water just below the oxycline of the meromictic Lake Suigetsu, Japan. Water samples were incubated with $^{13}$C-labeled bicarbonate under either light or dark conditions. The identity of active bicarbonate-fixing bacteria was revealed by 16S rRNA gene clone library analysis of $^{13}$C-labeled DNA fractions. Bacterial clones closely related to the green sulfur bacterial genus *Chlorobium* were detected under light conditions. Clone sequences phylogenetically affiliated with the genera *Arcobacter* and *Desulfocapsa* were also detected under light conditions. In the dark incubation, clones belonging to the class *Epsilonproteobacteria* were detected. Clone sequences belonging to a clade containing the genus *Thiomicrospira* were also retrieved from dark incubation samples. These results indicated that phylogenetically diverse bacteria might fix CO$_2$ in the water below the oxycline of Lake Suigetsu.

Key words: chemolithoautotrophy, green sulfur bacteria, primary producer

Introduction

Phototrophic sulfur bacteria use reduced sulfur compounds as electron donors during anoxygenic photosynthesis. They grow in opposing gradients of sulfide and oxygen in the presence of photosynthetically active radiation (Brune 1995, Sirevåg 1995). In the oxic-anoxic interface of meromictic lakes, phototrophic sulfur bacteria often form dense blooms (Van Gemerden & Mas 1995, Koizumi et al. 2004, Manske et al. 2005, Tonolla et al. 2005, Gregersen et al. 2009, Ng et al. 2010, Galand et al. 2012) and a high activity of bicarbonate fixation has been detected (Takahashi & Ichimura 1968, Van Gemerden & Mas 1995, and references therein). These studies suggest that phototrophic and chemotrophic sulfur bacteria may contribute to the carbon cycle through primary production in the anaerobic part of the water column. However, there is no direct evidence to support this suggestion.

Lake Suigetsu is a meromictic lake with an oxic-anoxic interface developed at a depth of 3–8 m, which separates the oxic low salinity mixolimnion from the anoxic saline sulfidogenic monimolimnion (Matsuyama & Saijo 1971, Kondo et al. 2000). Due to the entry of two different water types, i.e., freshwater through Lake Mikata and saltwater through Lakes Kugushi and Hiruga, Lake Suigetsu contains two layers of water with different densities. Thus, the deeper layers of the lake stagnate (Matsuyama 1973). With prolonged meromixis, the anoxic water chemistry of Lake Suigetsu is dominated by high concentrations (up to ca. 7 mM) of sulfide (Matsuyama & Saijo 1971, Kondo et al. 2000). Lake Suigetsu has attracted interest for studies of microbial sulfate reduction and sulfur oxidation in the carbon and sulfur cycles because of its hydrophysical characteristics, specifically the chemocline in the water column and the high concentration of sulfide in the monimolimnion. Thus, we used molecular methods to investigate the diversity and distribution of sulfate-reducing bacteria (Kondo et al. 2006, Kondo & Butani 2007) and phototrophic sulfur bacteria (Mori et al. 2010, 2013) as well as the entire bacterial community (Kondo et al. 2009). Matsuyama & Saijo (1971) and Takahashi & Ichimura (1968) reported...
that the maximum level of photosynthetic bicarbonate fixation took place in the chemocline, which suggests that phototrophic sulfur bacteria are involved in carbon assimilation in this region. However, oxygenic pico-sized cyanobacteria were detected at a maximum density of >10^6 cells mL^-1 even in the microaerobic and/or sulfidic chemocline of Lake Suigetsu (Okada et al. 2007). Sulfide-tolerant pico-sized cyanobacteria were also isolated from the chemocline of the lake (Ohki et al. 2012). These studies suggest that the cyanobacteria may also fix CO_2 in the chemocline. Furthermore, high rates of chemosynthetic (dark) bicarbonate assimilation have been reported in the chemocline of Lake Suigetsu during the winter season, which suggests that chemolithotrophic sulfur bacteria may act as bicarbonate fixers in the dark (Matsuyama & Saijo 1971). However, the active microorganisms in the chemocline of Lake Suigetsu remain unknown.

Stable isotope probing (SIP) is a method used for labeling microorganisms in cultivated or field samples, using a substrate enriched with a stable isotope. Various types of SIP focus on different biomarker molecules that become labeled via growth with the stable isotope substrate. These SIP methods include the labeling of membrane lipids, such as phospholipid-derived fatty acid (PLFA-SIP), deoxyribonucleic acid (DNA-SIP), and ribonucleic acid (RNA-SIP) (Neufeld et al. 2007a). The appeal of DNA-SIP is that it has the potential to retrieve labeled genomic DNA from the environment, which offers the opportunity to analyze purified DNA using a range of molecular techniques. The techniques of DNA-SIP that utilize ^13C-labeled substrates have been used to determine the active bacteria within the total bacterial community that contributes to particular metabolic pathways (Cébron et al. 2007, Glaubitz et al. 2009). In the present study, we identified the community composition of bicarbonate-fixing bacteria using DNA-SIP in the water just below the oxic–anoxic interface (oxycline) of Lake Suigetsu.

Materials and Methods

Sample collection and physicochemical analyses

Water samples were collected from 7 m depth (below the oxycline) in the central basin of Lake Suigetsu (35°35′N, 135°53′E) using a Van Dorn water sampler (Rigo Co. Ltd., Tokyo, Japan) on 13 March and 3 September 2012. Subsamples were sucked into a polyethylene bag (Lontainer, Sekisui Seikei Co. Ltd., Osaka, Japan) to avoid contact with air. Known volumes (1–50 mL depending upon sulfide concentrations) of subsamples to be used for sulfide analyses were fixed on site with a small spoonful of zinc acetate powder (approximately 0.2 g). All samples were kept in an ice-cooled box and brought to the laboratory within a few hours after sample collection.

The temperature and salinity were measured using a salinity-temperature recorder (Model 85, YSI, Yellow Spring, OH, USA). The dissolved oxygen (DO) concentration was measured using an oxygen meter (Hach, Loveland, CO, USA). The turbidity (as kation mg L^-1; ppm) was measured using a turbidity meter (Model PT-1, Alec Electronics Co., Ltd, Kobe, Japan). The photosynthetically active radiation (PAR) level was measured using an LI-192 underwater quantum sensor connected to an LI-1400 data logger (Li-Cor, Lincoln, NE, USA). The concentration of sulfide in the water below the oxycline was measured spectrophotometrically using the methylene blue method, as described by Kondo (2006).

Stable isotope probing incubations

Aliquots (1 L) of water samples were poured into 1,000-mL pressure culture bottles, and sodium sulfide solution (1 g L^-1 Na_2S·9H_2O) was added to adjust the sulfide concentration to environmental levels. The headspace was replaced with N_2. Sodium ^13C-bicarbonate (Cambridge Isotope Laboratories, Andover, MA, USA) was added to the 1 L water samples at a final concentration of 70–100 mg L^-1, which was approximately equal to the natural concentration of dissolved inorganic carbon (DIC) (Mori et al. 2013). The bottles were incubated in the light (2–3 μmol photons m^-2 s^-1) or dark at 10°C or 26°C for the March and September samples, respectively. Subsamples were collected using a needle and syringe at one or two-day intervals. The sulfide concentration in subsamples was measured using the methylene blue method (Kondo 2006). No sodium sulfide solution was added to the March samples, because sulfide concentration in the samples was constant through stable isotope probing (SIP) incubation. For the September samples, to maintain the sulfide concentration at environmental levels, an appropriate volume (0.2–0.3 mL) of sodium sulfide solution was added to the bottle under light conditions after two days incubation and then into the bottles under both light and dark conditions after four days incubation. Five or ten-day incubations were used to minimize changes in the bacterial community, while achieving significant ^13C-incorporation. The bacterial fraction in the water was collected by filtration onto a polycarbonate membrane filter (0.2 μm; Advantec, Tokyo, Japan) and was frozen at −30°C until further analyses.

DNA extraction

The bacterial cells on the filters were suspended in 10 mL of TNE buffer (10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 7.5). After the addition of 500 μL of 10% (w/v) sodium dodecyl sulfate solution and 100 μL of proteinase K solution (10 mg mL^-1), the filter was incubated overnight at 50°C with shaking. The lysates were extracted twice with phenol:chloroform:isoamyl alcohol (25 : 24 : 1, v/v/v) and once with chloroform:isoamyl alcohol (24 : 1, v/v). Subsequently, 10 mL of the aqueous phase was decanted into a 50 mL centrifuge tube. A one-fiftieth volume of 5 M NaCl and two volumes of 100% (v/v) ethanol were added to the tube. Precipitations were conducted overnight.
at −25°C. The samples were centrifuged at 2,380×g to collect the nucleic acids, washed with 70% ethanol, dried at room temperature, and dissolved in 1 mL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The concentration of the DNA solution was quantified using a NanoDrop Light spectrometer (Thermo scientific, Yokohama, Japan). DNA samples from *Chlorobium phaeovibrioides* DSM269 cultured with NaH¹³CO₃ or NaH¹²CO₃ were extracted using a FastDNA® Spin kit (MP Biomedicals) for use as size markers during density gradient fractionation.

### DNA-SIP gradient fractionation

Cesium chloride (CsCl) isopycnic ultracentrifugation and gradient fractionation was performed using the protocol of Neufeld et al. (2007b) with the minor modification of increasing the amount of DNA to 50 µg. The extracted DNA samples were centrifuged in 5.1 mL polyallomer quick-seal tubes (Beckman Coulter, Tokyo, Japan) in an NVT90 rotor (Beckman Coulter) using an XL-90 Ultracentrifuge (Beckman Coulter). Centrifugation was performed at 20°C for 40 h at 176,939×g. The ¹²C-DNA and ¹³C-DNA fractions were removed from the CsCl tube using a needle and syringe, as described in Neufeld et al. (2007b), and collected in 1.5 mL tubes. A marker tube containing *C. phaeovibrioides* DSM269 ¹²C-DNA and ¹³C-DNA was used alongside the CsCl tube as a visual guide. The DNA fractions were extracted three or four times with TE buffer-saturated 1-butanol. The DNA samples were precipitated with 20 µg glycogen and two volumes of polyethylene glycol (30% PEG 6,000 and 1.6 M NaCl). The pellet was washed with 70% ethanol and dissolved in 30 µL of TE buffer.

### 16S rDNA clone library

Clone libraries were generated from the DNA taken from ¹²C-DNA and ¹³C-DNA fractions. PCR amplification of the partial 16S ribosomal RNA gene (rDNA) was performed using the primers 8f (Amann et al. 1995) and 534r (Muyzer et al. 1993). PCR mixtures were prepared in a total volume of 50 µL, which contained 1×PCR buffer, 0.2 mM of each dNTP (Fermentas, Burlington, Canada), 0.4 µM of each primer, 2.5 U Taq DNA polymerase (Qiagen, Hilden, Germany), and 1 µL of template DNA. PCR amplification was conducted with the following conditions: initial denaturation at 94°C for 1 min, 25 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min, with a final elongation step of 7 min at 72°C. To confirm the PCR amplification, aliquots of the PCR products were separated by electrophoresis on a 2% (w/v) agarose gel with 1×TAE buffer (40 mM Tris-acetate, 1 mM EDTA; pH 8.0), stained with ethidium bromide, and visualized using a Quantity One GelDoc XR gel imaging system (Bio-Rad, Hercules, CA, USA). PCR products were cloned using a TA Cloning Kit (Invitrogen, Carlsbad, CA, USA) with the pCRII vector and *Escherichia coli* INVαF" competent cells, according to the manufacturer’s instructions. The transformed *E. coli* cells were sorted by blue-white selection after plating on Luria–Bertani plates containing 150 µg mL⁻¹ of ampicillin and 80 µg mL⁻¹ of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal). *Escherichia coli* containing the PCR product was cultured in Luria–Bertani broth containing 150 µg mL⁻¹ of ampicillin and plasmids were extracted from the cultured *E. coli* using a Wizard SV 96 Plasmid DNA purification system (Promega, Madison, WI, USA), according to the manufacturer’s instructions. The purified plasmids were sequenced using an ABI PRISM® 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The presence of chimeric sequences was checked with the DECIPHER’s Find Chimeras web tool (Wright et al. 2011).

### Evaluation of gradient fractionation of ¹³C-DNA from ¹²C-DNA

After removing the chimeras, 68–84 and 85–102 clones were obtained from the ¹²C-DNA and ¹³C-DNA fractions, respectively. The partial 16S ribosomal RNA gene (rDNA) sequences were aligned using the Silva SINA Webaligner subroutine of the Silva database (Pruesse et al. 2012; http://www.arb-silva.de/aligner/). To assess the successful separation of ¹²C-DNA from ¹³C-DNA by isopycnic ultracentrifugation, we statistically analyzed the differences between the 16S rDNA sequences retrieved from the ¹³C-DNA and ¹²C-DNA fractions using the LIBSHUFF in Mothur program (Schloss et al. 2009). The LIBSHUFF program reports two values, i.e., homologous [X] versus heterologous [Y] and Y versus X, where each represents the comparison of sequences in one library with the other. If one of these p values is <0.025, the populations are considered to be significantly different (Eisenlord & Zak 2010).

### Phylogenetic analyses and sequence population diversity

The sequences of the clone library constructed from the ¹³C-labeled bacterial DNA with similarities >97% were considered to represent the same operational taxonomic unit (OTU) using the software Mothur (Schloss et al. 2009). Representative clone sequences of OTUs were picked at random and homology searches against the GenBank/EMBL/DBJ sequence databases were performed using the FASTA program (Kanehisa et al. 2002). Representative clone sequences were aligned with reference sequences obtained from the GenBank/EMBL/DBJ databases using the Silva SINA Webaligner subroutine of the Silva database. Neighbor-joining analysis was performed using the MEGA software package, version 4 (Tamura et al. 2007). The confidence limits of the tree topologies were estimated using 1,000 bootstrap replicates (Felsenstein 1985).

### Nucleotide sequence accession numbers

The 16S ribosomal RNA gene sequences determined in this study were deposited in the DDBJ under accession
numbers AB896820–AB897482.

Results

Physicochemical parameters

A summary of the vertical profiles of the physicochemical properties in the central basin of Lake Suigetsu on the sampling dates is shown in Fig. 1. A thermocline was evident between 5 m and 10 m (Fig. 1A). A steep salinity gradient developed in March (6 m to 10 m) and a gradual salinity gradient developed in September (2 m to 10 m) (Fig. 1B). The dissolved oxygen (DO) concentration decreased with water depth (Fig. 1C). The depth of the oxycline varied from 6 to 7 m and 2 to 4 m in March and September, respectively. Especially in September, the DO concentration declined gradually from 4 m to 7 m depth. The DO concentrations at 7 m were 0.2 mg L$^{-1}$ and 0.0 mg L$^{-1}$ in March and September, respectively. The turbidity peaked at 7 m and 6 m depth in March and September, respectively (Fig. 1D). The photosynthetically active radiation (PAR) levels decreased with water depth and were 2.64 µmol photons m$^{-2}$ s$^{-1}$ and 2.94 µmol photons m$^{-2}$ s$^{-1}$ at a depth of 7 m in March and September, respectively (Fig. 1E). The sulfide concentrations at 7 m were 0.64 mM and 0.15 mM in March and September, respectively.

Assessment of separation between $^{12}$C- and $^{13}$C-labeled DNA by density gradient ultracentrifugation

Stable isotope probing (SIP) was carried out to investigate the composition of the CO$_2$-fixing bacterial community in the water just below the oxycline. Eight 16S ribosomal RNA gene (rDNA) libraries were constructed with $^{12}$C-DNA and $^{13}$C-DNA from the four SIP incubation experiments. To assess the successful separation of $^{13}$C-DNA from $^{12}$C-DNA by isopycnic ultracentrifugation, we performed a statistical analysis of the differences between 16S rDNA sequences retrieved from the $^{13}$C-DNA and $^{12}$C-DNA fractions using the LIBSHUFF program. In the pairwise comparisons, the community compositions from the $^{12}$C-DNA and $^{13}$C-DNA fractions differed significantly (Table 1).

Phylogenetic composition of CO$_2$-fixing bacteria

A total of 363 clones from the $^{13}$C-DNA fractions were sorted into 49 operational taxonomic units (OTUs) based on our definition of >97% sequence identity (Table 2). The majority of the sequences belonged to the phylum Chlorobi and the classes Deltaproteobacteria, Gammaproteobacteria, and Epsilonproteobacteria (Fig. 2). In the samples incubated under light conditions, OTU36, OTU18 and OTU10 accounted for 26%, 45% and 24% of the March library, respectively (Table 2). Clones from OTU36 were closely related to Chlorobium phaeovibrioides of the phylum Chlorobi (98.7% identity) (Fig. 2B and Table 2). Clones from OTU18 phylogenetically clustered with the genus Desulfocapsa and clones from OTU10 belonged to the clade including the genus Arcobacter (Figs. 3A and 3B). Clones from OTU33 comprised 60% of the total in the September sample incubated under light conditions and they were closely related to Chlorobium luteolum (97.7% identity) (Table 2). No clones belonging to the phylum Cyanobacteria were detected in the samples incubated under light conditions (Table 2). In the samples incubated under dark conditions, clone sequences from OTU28 accounted for 24% of the March library (Table 2). Phylogenetic analysis indicated OTU28 belonged to the genus Thiomicrospira (Fig. 3C). Clones of OTU8, phylogenetically belonging to the class Epsilonproteobacteria, comprised 39% of the total in the September sample incubated under dark conditions (Table 2, Fig. 3D). Surprisingly, the green sulfur bacterial genus Chlorobium accounted for 48% and 18% of the March and September libraries, respectively (Table 2).

Discussion

Using stable isotope probing (SIP), we analyzed the ac-
Table 2. Nucleotide sequence identity of representative 16S ribosomal RNA gene (rDNA) fragments affiliated with the operational taxonomic units (OTU) and the number of the clones grouped into the OTU.

| OTU | No. of clones | March          | September       | Most similar 16S rRNA gene sequence in NCBI based on FASTA search | Identity (%) |
|-----|--------------|----------------|-----------------|---------------------------------------------------------------|--------------|
|     |              | Light | Dark | Light | Dark | Sulfurimonas paradoxinella strain GO25 (NR_041439) | 92.0         |
| 1   | 1            |       |      |       |      | Campylobacterium bacterium SAM4 (AM884342)         | 93.0         |
| 2   | 1            |       |      |       |      | Sulfurimonas autotrophica DSM 16294 (CP002205)    | 93.3         |
| 3   | 2            | 4     |      |       |      | Sulfurimonas denitrificans DSM 1251 (CP000153)    | 95.1         |
| 4   | 1            |       |      |       |      | Uncultured bacterium clone SeaWat_G729 (JQ195635) | 93.9         |
| 5   | 2            | 2     |      | 37-8  | 32   | Sulfurimonas sp. 37-8 (AB235320)                | 92.7         |
| 6   | 1            |       |      |       |      | Uncultured bacterium clone SeaWat_G729 (JQ195635) | 93.5         |
| 7   | 14           | 33    |      |       |      | Thioreductor micantisoli BKB25Ts-Y (NR_041022)    | 86.9         |
| 8   |              |       |      |       |      | Thioreductor micantisoli (AB175498)            | 86.4         |
| 9   | 24           |       |      |       |      | Arcobacter thereius strain 16389 (AY314753)      | 90.0         |
| 10  | 6            |       |      | 16695-3 | 32   | Arcobacter thereius strain 16695-3 (AY314754)   | 92.9         |
| 11  |              |       |      |       |      | Arcobacter nitrofigilis strain F2173 (EU106661)   | 93.6         |
| 12  |              |       |      |       |      | Arcobacter marinus strain CL-S1 (EU512920)       | 95.7         |
| 13  |              |       |      |       |      | Uncultured bacterium clone SeaWat_G729 (JQ195635) | 93.9         |
| 14  |              |       |      |       |      | Thioreductor micantisoli (AB175498)            | 86.4         |
| 15  |              |       |      |       |      | Desulfobacterium anilini DSM 4660 (AJ237601)      | 88.8         |
| 16  |              |       |      |       |      | Desulfobacterium autotrophica DSM 3882 (AJ237602) | 95.5         |
| 17  |              |       |      |       |      | Desulfocapsa sulfexigens DSM 10523 (CP003985)    | 90.4         |
| 18  | 46           | 1     | 1    | 2     | 2    | Desulfocapsa sulfexigens DSM 10523 (CP003985)    | 93.0         |
| 19  |              |       |      |       |      | Desulfocapsa sulfexigens DSM 10523 (CP003985)    | 97.8         |
| 20  |              |       |      |       |      | Desulfocapsa sulfexigens DSM 10523 (CP003985)    | 100          |
| 21  |              |       |      |       |      | Rhodobacteraceae bacterium 270 (AJ810839)         | 97.2         |
| 22  |              |       |      |       |      | Shewanella sp. S2 (FJ589033)                      | 100          |
| 23  |              |       |      |       |      | Shewanella baltica OSM678 (CP002383)              | 99.6         |
| 24  |              |       |      |       |      | Methylococcaceae bacterium R-49797 (HF58990)      | 96.9         |
| 25  |              |       |      |       |      | Halochromatium sp. AR2202 (AJ401219)              | 99.4         |
| 26  |              |       |      |       |      | Thiomicrospira chilensis strain Ch-1 (NR_028680)  | 88.2         |
| 27  |              |       |      |       |      | Thiomicrospira sp. NP20 (EU196336)                | 93.1         |
| 28  |              |       |      |       |      | Thiomicrospira sp. NP20 (EU196336)                | 95.1         |
| 29  |              |       |      |       |      | Thiomicrospira sp. UST724 (KC354369)              | 96.1         |
| 30  |              |       |      |       |      | Thiomicrospira sp. Art-3 (AF013973)               | 97.3         |
| 31  |              |       |      |       |      | Chlorobium limicola (AB517714)                    | 99.6         |
| 32  |              |       |      |       |      | Chlorobium luteolum DSM 273 (NR_074096)          | 97.7         |
| 33  |              |       |      | 2     | 23   | Chlorobium luteolum DSM 273 (NR_074096)          | 96.6         |
| 34  |              |       |      |       |      | Chlorobium luteolum DSM 273 (NR_074096)          | 97.5         |
| 35  |              |       |      |       |      | Chlorobium sp. sy9 (EU770420)                     | 97.5         |
| 36  | 27           | 15    | 7    |       |      | Chlorobium phaeovibrioides DSM 265 (CP000607)     | 98.7         |
| 37  |              |       |      |       |      | Salinirepens amamiensis (AB517714)               | 93.4         |
| 38  |              |       |      |       |      | Sphingomonas sp. BAC84 (EU131006)                | 99.2         |
| 39  |              |       |      |       |      | Uncultured bacterium clone NZ_309 Bac31 (JF268362) | 97.7         |
| 40  |              |       |      |       |      | Uncultured bacterium clone JSC 140 (KF624500)     | 91.3         |
| 41  |              |       |      |       |      | Uncultured bacterium clone SINP973 (HM127843)     | 100          |
| 42  |              |       |      |       |      | Geitlerinema sp. PCC 7407 (NR_102448)             | 86.0         |
| 43  |              |       |      |       |      | Cyanobium sp. Suigetsu-CR5 (AB610889)             | 99.8         |
| 44  |              |       |      |       |      | Uncultured bacterium clone BMS3AB13 (AB858514)    | 80.3         |
| 45  |              |       |      |       |      | Uncultured actinobacterium clone BAC K005 (GQ387490) | 91.7         |
| 46  |              |       |      |       |      | Micrococcus sp. TVS41 (KF142392)                  | 89.5         |
| 47  |              |       |      |       |      | Uncultured bacterium clone SeaWat_71158 (JQ198155) | 99.4         |
| 48  |              |       |      |       |      | Uncultured bacterium clone SINN1125 (HM128507)    | 76.6         |
| 49  |              |       |      |       |      | Uncultured bacterium clone SINI551 (HM126929)     | 93.4         |

1) Number of 16S ribosomal RNA gene (rDNA) clones obtained from the 13C-DNA fractions of the stable isotope probing microcosms constructed with the water samples from below the oxycline of Lake Suigetsu.

2) The characters in parentheses indicate accession numbers.
tive CO$_2$-fixing bacterial population in the water just below the oxycline of Lake Suigetsu. In Lake Suigetsu, green sulfur bacteria and cyanobacteria are thought to be major carbon fixers (Okada et al. 2007, Kondo et al. 2009, Ohki et al. 2012, Mori et al. 2013). Our SIP incubation, in which the environmental conditions were simulated, indicated that green sulfur bacteria might fix CO$_2$ by photosynthesis, while cyanobacteria might not act as primary producers in the water below the oxycline of Lake Suigetsu. Clones closely related to Chlorobium luteolum and Chlorobium phaeovibrioides were dominant in the water incubated under light conditions, and accounted for 86% and 93% of the green sulfur bacterial clones in September and March, respectively (Table 2). In our previous study, C. luteolum and C. phaeovibrioides were shown to be dominant green sulfur bacteria in the chemocline of Lake Suigetsu in the summer and winter seasons, respectively.

Abundant green sulfur bacterial clones belonging to the genus Chlorobium were also recovered from the water incubated under dark conditions. Green sulfur bacterial species have no capacity for chemolithoautotrophic growth (Kämpf & Pfennig 1980). It is difficult to explain the reason for the detection of green sulfur bacteria in the dark conditions. Green sulfur bacteria capable of assimilating CO$_2$ using thermal blackbody radiation have been isolated from a deep-sea hydrothermal field (Beatty et al. 2005). However, it is highly unlikely that blackbody radiation (wavelengths in excess of 700 nm) was present during our incubation, or below the oxycline in Lake Suigetsu, because there were no thermal sources. A possible explanation for the dark carbon assimilation by green sulfur bacteria is cross-feeding, which is considered to be a ma-
CO₂-fixing bacteria in Lake Suigetsu

The long incubation time increases the risk of cross-feeding of ¹³C from the primary consumers to the rest of the community (Dumont & Murrell 2005). In this study, we used 5 or 10 days incubation time to make a clear distinction between the ¹³C-DNA and ¹²C-DNA fractions, which means that we cannot exclude the possibility of cross-feeding. Some green sulfur bacterial species can also assimilate low-molecular-weight organic acids such as acetate and pyruvate in the presence of CO₂ and sulfide (Imhoff 1995). In general, low-molecular-weight organic acids may be produced by bacterial decomposition of organic matter in anaerobic environments. Clones related to the heterotrophic bacterial genus *Shewanella* and clones belonging to the heterotrophic bacterial phylum *Bacteroidetes* were detected from the ¹³C-DNA fraction in the water samples incubated in the dark (Table 2 and Fig. 2B). These heterotrophic bacteria may ferment the ¹³C-labeled organic compounds, which were produced by chemolithotrophic bacteria belonging to OTU8 and OTU28, and produce low-molecular-weight organic acids such as acetate and pyruvate. Green sulfur bacterial clones detected in the dark incubation might assimilate the low-molecular-weight organic acids that were produced by fermentation bacteria.

Clones from OTU8 dominated in the September sample incubated under dark conditions (Table 2). Our molecular characterization showed that clone sequences from OTU8 belonged to the class *Epsilonproteobacteria* in various sulfur-rich environments such as a cave sulfidic spring, a hydrothermal vent, a redox cline in the stratified ocean and an iron seep area (Engel et al. 2004, Nakagawa et al. 2005, Grote et al. 2008, Haaijer et al. 2008). Some bacteria belonging to the class *Epsilonproteobacteria* grow chemolithotrophically using sulfur compounds as energy sources and/or electron acceptors (Nakagawa and Takaki 2009). Results of further efforts are awaited, including a more detailed phylogeny, isolation, and information on the potential for sulfur reduction by these OTU8-affiliated bacteria.

Clones from OTU28 dominated only in the March sample incubated under dark conditions (Table 2). Clone sequences from OTU27, 30 and 31 were detected in the September sample (Table 2). Phylogenetic analyses indicated...
that these clone sequences belonged to the genus *Thiomicrospira* (Fig. 3C). Members of the genus *Thiomicrospira* are strictly aerobic and grow chemolithotrophically using thiosulfate, tetrathionate, and sulfur as electron donors (Knittel et al. 2005). One possible reason for their detection from the anoxic incubation is contamination by oxygen into the water sample during SIP incubation. However, this possibility seems unlikely, because decreases in the sulfide concentration were insignificant throughout the SIP incubation. Alternatively, micro-aerobic oxycline water might have contaminated the water samples for SIP incubation during water sampling. We speculate that clones belonging to the genus *Thiomicrospira* fixed CO$_2$ under micro-aerobic conditions at the start of the SIP incubation. Matsuyama & Saijo (1971) indicated that chemosynthetic activity in the chemocline in the winter season is more than four times as high as that in the summer season, and that chemosynthetic activity is higher than photosynthetic activity in the winter season. They suggested that aerobic sulfur-oxidizing bacteria contributed to the high levels of dark CO$_2$ fixation. The results of our SIP study, showing the dominance of the genus *Thiomicrospira*, support their suggestion.

Clones from OTU18 fixed CO$_2$ especially in the March samples incubated under light conditions (Table 2). Our molecular characterization showed that clone sequences from OTU18 clustered with bacterial sequences of the genus *Desulfocapsa* (Fig. 3A). *Desulfocapsa sulfoexigens* and *Desulfocapsa thiozymnogenes* grow anaerobically using bicarbonate as the sole carbon source via the disproportionation of elemental sulfur, sulfite, and thiosulfate in the presence of a hydrogen-sulfide scavenging agent (Janssen et al. 1996, Finster et al. 1998). In the chemocline of meromictic Lake Cadagno, an aggregation of sulfur-disproportionating bacteria and purple sulfur bacteria was identified using in situ hybridization with specific oligonucleotide probes (Peduzzi et al. 2003). They suggested synergistic interactions that presumably resemble a source-sink relationship for sulfide between sulfur-disproportionating bacteria and the purple sulfur bacteria acting as biotic scavengers. In our SIP study, green sulfur bacterial clones belonging to OTU36 dominated in the water incubated under light conditions. Green sulfur bacteria oxidize sulfide and accumulate elemental sulfur on their cell surfaces (Frigaard & Dahl 2009). These green sulfur bacteria may act as hydrogen-sulfide scavengers and supply elemental sulfur to bacteria belonging to OTU18 under light conditions.

Clones from OTU10, phylogenetically related to the genus *Arcobacter*, incorporated $^{13}$C only in the March sample incubated under light conditions (Table 2 and Fig. 3B). Various bacteria belonging to the genus *Arcobacter* have been reported from both animal and environmental sources (McClung et al. 1983, Wirsen et al. 2002, Donachie et al. 2005). An oilfield bacterium FWKO B, closely related to members of the genus *Arcobacter*, and *Candidatus Arcobacter sulfidicus* have been reported to be sulfide-oxidizing autotrophs (Gevertz et al. 2000, Wirsen et al. 2002). Bacteria belonging to OTU10 may have fixed CO$_2$ and oxidized sulfide in the water below the oxycline in March. These clones were only found in the light incubation, although phototrophic bacteria belonging to the genus *Arcobacter* were not found. We speculate that clones belonging to OTU10 might incorporate CO$_2$ and oxidize sulfide, which was produced by sulfur-disproportionating bacterial clones belonging to OTU18 that dominated in the sample incubated under light conditions.

In this study, we used SIP analysis to detect carbon-assimilating microorganisms in the water just below the oxycline of the meromictic Lake Suigetsu. The green sulfur bacterial genus *Chlorobium* may play a role in photolithotrophic carbon fixation below the oxycline, where the water was rich in reduced sulfur compounds and photo-synthetically active radiation was present. Phylogenetically diverse bacteria may also fix CO$_2$ by chemolithotrophy. Interestingly, our SIP study indicated the possibility that bacteria belonging to the genera *Chlorobium* and *Arcobacter* may act not only as primary producers but also as sulfide scavengers and may supply elemental sulfur or consume sulfide from sulfur-disproportionating *Desulfocapsa* in the water below the oxycline of Lake Suigetsu.

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