Comprehensive Detection of Phototrophic Sulfur Bacteria Using PCR Primers That Target Reverse Dissimilatory Sulfite Reductase Gene

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A new set of primers for the detection of phototrophic sulfur bacteria in natural environments is described. The primers target the α -subunit of the reverse dissimilatory sulfite reductase gene (*dsrA*). PCR-amplification resulted in products of the expected size from all the phototrophic strains tested, including purple sulfur and green sulfur bacteria. Seventy-nine clones obtained from environmental DNA using the primers were sequenced and all found to be closely related to the *dsrA* of purple sulfur bacteria and green sulfur bacteria. This newly developed PCR assay targeting *dsrA* is rapid and simple for the detection of phototrophic sulfur bacteria *in situ* and superior to the use of culture-dependent techniques.

Key words: meromictic lake, phototrophic sulfur bacteria, reverse dissimilatory sulfite reductase

Phototrophic sulfur bacteria constitute a paraphyletic group of metabolically diverse anaerobes capable of utilizing light as an energy source, but that do not evolve molecular oxygen. Widely distributed in nature where oxygen is either absent or present at low concentrations and opposing gradients of sulfide and light exist simultaneously, phototrophic sulfur bacteria use sulfide or other reduced sulfur compounds as electron donors in photosynthesis, and play an important role in sulfur cycling in aquatic systems such as lacustrine environments, where anoxic layers containing reduced sulfur compounds are exposed to light (19). These bacteria supply fixed carbon to chemoorganotrophic organisms, and thus, also have a significant role in the carbon cycle in holomictic and meromictic lakes (26, 35).

Phototrophic sulfur bacteria are traditionally classified as purple sulfur bacteria within the *Gammaproteobacteria* and as green sulfur bacteria within the phylum *Chlorobi* (26, 35). Their polyphyletic nature restricts the concomitant detection of all recognized members using a single 16S rRNA gene-targeting probe or primer set in environmental analyses (1, 3, 8, 28, 34). Alternative approaches have been developed to detect phototrophic sulfur bacteria using PCR primers that target functional genes coding for key enzymes of sulfur oxidation pathways; ATP sulfurylase, the iron-sulfur flavo-protein adenosine-5'-phosphosulfate (APS) reductase and siroheme sulfite reductase (18, 20, 21).

Dissimilatory sulfite reductase (DsrAB) is a key enzyme in dissimilatory sulfate reduction in sulfate-reducing prokaryotes. The presence of DsrAB with a proposed function in sulfide oxidation has been demonstrated for colorless and purple sulfur bacteria (29, 30). Analyses of genome data revealed the presence of the dissimilatory sulfite reductase genes (*dsrAB*) in phototrophic sulfur bacteria (e.g. reference 22). Moreover, *dsrAB* sequences of phototrophic sulfur bacteria were conserved and phylogenetically distinct from

sulfate-reducing prokaryotes (4). Thus, *dsrAB* coding for the reverse-operating DsrAB is a potential marker gene for phototrophic sulfur bacteria unlike primers and probes targeted to the 16S rRNA gene (7). Recently, PCR assays have been developed for the amplification of *dsrAB* from cultures and natural environments (18). However, these researchers still used multiple sets of primers to amplify the *dsrAB* from the phototrophic sulfur bacteria.

This paper reports the development of new PCR primers for the rapid and sensitive detection of *dsrA* in phototrophic purple and green sulfur bacteria simultaneously and an analysis of bacterial communities from natural environments. The primers were tested using water samples from a meromictic lake, where phototrophic sulfur bacteria often form dense blooms in and/or just below the oxycline during the summer (17, 23).

Materials and Methods

Bacterial strains

The following strains of phototrophic sulfur bacteria were obtained from Deutscher Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) either as lyophilized or actively growing cultures: *Allochrochromatium vinosum* DSM 180^T, *Halorhodospira halophila* DSM 244^T, *Halochromatium salexigens* DSM 4395^T, *Thiobaca trueperi* DSM 13587^T, *Thiocapsa marina* DSM 5653^T, *Thiorhodococcus drewsii* DSM 15006, *Marichromatium gracile* DSM 203^T, *Thiococcus pfennigii* DSM 266, *Prosthecochloris vibrioformis* DSM 261 *Chlorobium limicola* DSM 245^T, *Chlorobium luteolum* DSM 273^T, *Prosthecochloris aestuarii* DSM 271^T, and *Chlorobium phaeovibrioides* DSM 269^T. DNA was directly extracted from the lyophilized cells or cell pellets harvested from actively growing cultures by centrifugation using FastDNA Spin Kit (MP Biomedicals, Solon, OH, USA). DNA from *Chloroflexus auranticus* DSM 635^T, *Heliobacterium chlorum* DSM 3682^T, *Thiobacillus denitrificans* DSM 12475^T, *Thiomicrospira pelophila* DSM 1534^T, *Desulfotomaculum geothermicum* DSM 3669^T, *Desulfovibrio desulfuricans* DSM 642^T, *Desulfovibrio africanus* DSM 2603^T, *Desulfomicrobium norvegicum* DSM 1741^T, *Desulfobacter postgatei* DSM 2034^T, *Desulfobulbus propionicus* DSM 2032^T, *Desulfobulbus* sp. DSM 2033, *Desulfosarcina variabilis*

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DSM 2060^T, *Desulfosporocinas orientis* DSM 765^T, *Alteromonas macleodii* IAM 12920^T, *Shewanella putrefaciens* IAM 1509^T, *Flavobacterium aquatile* IAM 12316^T, and *Cytophaga matinoiflava* IAM 14170^T was extracted as described previously (9, 11, 12, 27) and used as negative controls in PCR experiments.

Sample collection and DNA extraction

Water samples were collected from the central basin of the meromictic Lake Suigetsu (35°35'N, 135°53'E) on 21st May 2008 using a Kitahara water sampler (Rigo-sha, Saitama, Japan). The samples were immediately added to an autoclaved BOD bottle to prevent contact with air. After transportation to the laboratory, within a few hours of sampling, a 50-mL aliquot was filtered through a sterile polycarbonate membrane filter (0.2 µm; Toyo Roshi Kaisha, Tokyo, Japan) to collect the microbial biomass for subsequent nucleic acid purification. Nucleic acids were extracted from the filtered samples using a FastDNA Spin Kit as described previously (16).

Sequencing of *dsrAB* genes from phototrophic sulfur bacteria

A multiple alignment of purple and green sulfur bacterial *dsrAB* sequences, which have been deposited in the EMBL/GenBank/

DDBJ, was constructed using the Clustal X program (33). Based upon the alignment, appropriate sites for *dsrAB*-targeting PCR primers were designed to amplify the nearly entire length of *dsrAB*. PCR amplification of an *dsrAB* fragment using the newly designed primers was performed with the following thermal cycling conditions: initial denaturation at 94°C for 1 min, 30 cycles of 94°C for 30 s, 65°C for 30 s and 72°C for 30 s, and a final elongation step at 72°C for 7 min with a thermocycler (MJ Mini; Bio-Rad, Hercules, CA, USA), except that the annealing temperature for the primer set for green sulfur bacterial strains was 50°C. PCR was carried out in a 50 µL volume containing 2.5 U of *Taq* DNA polymerase (Qiagen, Hilden, Germany), 1× PCR buffer, 200 µM dNTP (Fermentas, Burlington, Canada), 0.4 µM of each primer, and 1 µL of template DNA. The presence and size of the PCR products were analyzed by electrophoresis on a ethidium bromide-stained 1% (w/v) agarose gel in a 1× TAE (40 mM Tris-acetate, 1 mM EDTA [pH 8.0]) and visualized on a Quantity One GelDoc XR gel imaging system (Bio-Rad). The products were cloned using the TA Cloning[®] Kit (Invitrogen, Carlsbad, CA, USA) for sequencing of the 5' and 3' ends of the gene. Cloned inserts were reamplified using the vector primers M13 forward and reverse (25 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s) and the resulting PCR products were

Table 1. *dsr* alignment showing the targets of the PGdsrAF and PGdsrAR primers corresponding to the *Allochrochromatium vinosum* DSM 180^T *dsrAB* sequence at positions 379–402 and 850–870 from the 5' end of its coding region, respectively. Periods (.) indicate identity with primer sequence.

Microorganisms	Strain no	PGdsrAF	PGdsrAR
Primer		5' -CAYGGBACAGACCGGBRAYATYATG-3'	5' -RCAGTGCATRCACGHCACRCA-3'
Target		5' -CAYGGBACAGACCGGBRAYATYATG-3'	3' -YGTACGCTAYGTMGCDTGYGT-5'
Purple sulfur bacteria			
<i>Allochrochromatium vinosum</i>	DSM180 ^T		
<i>Halochromatium salexigens</i>	DSM4395 ^T		
<i>Halorhodospira halophila</i>	DSM244 ^T		
<i>Marichromatium gracile</i>	DSM203 ^T		
<i>Thiorhodococcus drowsii</i>	DSM15006 ^T		
<i>Thiobaca trueperi</i>	DSM13587 ^T		
<i>Thiocapsa marina</i>	DSM5653 ^T		
<i>Thiocystis gelatinosa</i>	DSM215 ^T		
Green sulfur bacteria			
<i>Chlorobium phaeobacteroides</i>	DSM266		
<i>Chlorobaculum tepidum</i>	DSM12025 ^T		
<i>Chlorobium limicola</i>	DSM257		
<i>Chloeobium luteorum</i>	DSM273 ^T		
<i>Prosthecochloris aestuarii</i>	DSM271		
<i>Chlorobium phaeovibrioides</i>	DSM261		
Colorless sulfur bacteria			
<i>Thiobacillus denitrificans</i>	DSM12475 ^T	T.....	T.....
<i>Thiothrix nivea</i>	DSM5205 ^T		
Sulfate-reducing prokaryote			
<i>Archaeoglobus fulgidus</i>	DSM4304 ^T	TC...T...A.....C	AC.....
<i>Thermodesulfobacterium commune</i>	DSM2178 ^T	TCT.....C	G.ACAA...
<i>Thermodesulfobacterium islandicus</i>	DSM12570 ^T	TCT...A.....GG.T	...T.....A...T...
<i>Desulfobulbus propionicus</i>	DSM2032 ^T	TCC.....G..C	CCA...
<i>Desulfotobacterium dehalogenans</i>	DSM9161 ^T	AGCT...T...A...GC...C	...T.....CCA...
<i>Desulfococcus multivorans</i>	DSM2059 ^T	TCC.....C	C.....
<i>Desulfotomaculum acetoxidans</i>	DSM771 ^T	TCT.....T	T...CA...
<i>Desulfosporosinus orientis</i>	DSM765 ^T	ATCA...A...A.....T	...T.....T...CA...
<i>Desulfosarcina variabilis</i>	DSM2060 ^T	TCC.....T	CCA...
<i>Desulfovibrio africanus</i>	DSM2603 ^T	TCC.....G..	
<i>Desulfovibrio desulfuricans</i>	DSM642 ^T	TTCC.....T.GT.	...T.....C.....
<i>Desulfobacter postgatei</i>	DSM2034 ^T	GCC.....T	...T.....CCA...
<i>Desulfotomaculum ruminus</i>	DSM2154 ^T	GCC...G.....GG.T	T...CCA...

purified using the Wizard[®] SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Sequencing was performed for 4 PCR clones and the purified PCR products from each strain using a *Taq* DyeDeoxy[™] Terminator Cycle Sequencing Kit and ABI Prism 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Design of PCR primers targeting phototrophic sulfur bacteria

A multiple alignment of *dsrAB* sequences determined in this study and retrieved from DNA databases, was constructed with Clustal X (33), and appropriate sites in the *dsrA* gene were chosen for use in PCR primers to selectively detect phototrophic sulfur bacteria. The newly designed primers were PGdsrAF and PGdsrAR, corresponding to the *Allochrochromatium vinosum* DSM 180^T *dsrAB* sequence at positions 379–402 and 850–870 from the 5' end of the coding region, respectively (Table 1). Primer specificity, with reference to published sequences, was analyzed using the FASTA program (25) at DDBJ.

PCR was carried out in 50 µL containing 1× PCR buffer, 200 µM of each dNTP, 0.4 µM of each primer, 2.5 U of *Taq* DNA polymerase, and 1 µL of template DNA. Amplification was performed in the MJ Mini thermal cycler as follows: initial denaturation at 94°C for 1 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s, and a final elongation step of 7 min at 72°C. Aliquots of the PCR products were analyzed by electrophoresis on a 1% (w/v) agarose gel in 1× TAE buffer. Ethidium bromide-stained bands were digitally recorded using the GelDoc XR gel imaging system.

Clone library-based analysis

After PCR amplification of the DNA retrieved from water samples of Lake Suigetsu, unpurified *dsrA* PCR products were cloned using the TA Cloning[®] Kit according to the manufacturer's instructions. Cloned inserts were reamplified using the vector primers M13 forward and reverse (25 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s) and the resulting PCR products were purified using the Wizard SV Gel and PCR Clean-Up System according to instructions. Randomly selected clones were sequenced as mentioned above.

Phylogenetic and statistical analyses

Partial *dsrA* sequences were aligned using Clustal X (33). The Neighbor-joining (NJ) analysis was performed using the MEGA software package, version 4 (32). Confidence limits for the tree's

topology were estimated using a bootstrap analysis of 1,000 replications (5).

Sequences were grouped into operational taxonomic units (OTUs) using the software DOTUR (30). Here, clones with a sequence similarity of greater than 88% were considered to represent the same OTU. Coverage (*C*) was calculated using the following formula $C=1-(n_i/N)$, where n_i is the number of OTUs that occurred only once in the clone library and *N* is the total number of clones analyzed (6).

Nucleotide sequence accession numbers

Partial *dsrAB* sequence data on cultured strains determined in this study have been deposited in the DDBJ, under accession numbers AB461934 to AB461940. Partially cloned *dsrA* sequences recovered from the water samples of Lake Suigetsu were deposited in DDBJ under accession numbers AB544492 to AB544570.

Results and Discussion

Design and evaluation of PCR primers

dsrAB sequences of reference phototrophic sulfur bacteria, which are available from the DDBJ/EMBL/GenBank databases, were aligned with Clustal X (33). From these aligned sequences, at least two consensus sequences were retrieved in the *dsrA* region, PGdsrAF and PGdsrAR. Homology searching using a FASTA program (25) indicated that while PGdsrAR matched *dsrA* sequences of phototrophic sulfur bacteria it also matched *dsrA* of some colorless sulfur bacteria and sulfate-reducing bacteria. PGdsrAF matched only *dsrA* sequences from phototrophic sulfur bacteria though not *Thiothrix nivea* DSM 5205^T. We therefore chose these two regions for use as PCR primers to selectively detect the *dsrA* of phototrophic sulfur bacteria. PCR amplification of the partial *dsrA* using the primers PGdsrAF and PGdsrAR resulted in products of the predicted size (*ca.* 490 bp) from all strains tested (Fig. 1), which encompassed almost all known purple and green sulfur bacteria. No amplification could be observed with DNA from sulfate-reducing bacteria except for *Desulfovibrio desulfuricans* DSM 642^T and *Desulfovibrio africanus* DSM 2603^T. *D. africanus* DSM

Fig. 1. PCR amplification of 16S rRNA gene from representative phototrophic sulfur bacteria, and sulfur-oxidizing, sulfate-reducing, and heterotrophic bacteria as controls using a set of bacterial universal primers (A) and of *dsrA* using PGdsrAF and PGdsrAR specific to phototrophic sulfur bacteria (B) analyzed by 1% (w/v) agarose gel electrophoresis in 1× TAE stained with ethidium bromide. Lanes: M, EZ Load[™] 100 bp PCR Molecular Ruler (Bio-Rad); 1, *Allochrochromatium vinosum* DSM 180^T; 2, *Halochrochromatium salexigens* DSM 4395^T; 3, *Halorhodospira halophila* DSM 244^T; 4, *Thiobaca trueperi* DSM 13587^T; 5, *Thiocapsa marina* DSM 5653^T; 6, *Thiococcus pfennigii* DSM 226; 7, *Thiorhodococcus drewsii* DSM 15006^T; 8, *Chlorobium limicola* DSM 245^T; 9, *Chlorobium. luteolum* DSM 273^T; 10, *Chlorobium. phaeovibrioides* DSM 269^T; 11, *Prosthecochloris aestuarii* DSM 272; 12, *P. vibrioformis* DSM 261; 13, *Chloroflexus aurantiacus* DSM 635^T; 14, *Helio bacterium chlorum* DSM 3682^T; 15, *Thiobacillus denitrificans* DSM 12475^T; 16, *Thiomicrospira pelophila* DSM 1534^T; 17, *Desulfotomaculum geothermicum* DSM 3669^T; 18, *Desulfovibrio desulfuricans* DSM 642^T; 19, *Desulfovibrio africanus* DSM 2603^T; 20, *Desulfomicrobium norvegicum* DSM 1741^T; 21, *Desulfobacter postgatei* DSM 2034^T; 22, *Desulfobulbus* sp. DSM 2033; 23, *Desulfobulbus propionicus* DSM 2032^T; 24, *Desulfosarcina variabilis* DSM 2060^T; 25, *Desulfosporocinas orientis* DSM 765^T; 26, *Alteromonas macleodii* IAM 12920^T; 27, *Shewanella putrefaciens* IAM 1509^T; 28, *Flavobacterium aquatile* IAM 12316^T; 29, *Cytophaga matinoiflava* IAM 14170^T; and C, no template control.

2603^T has the same sequence in the PGdsrAR region. No amplification was observed for any of the non-sulfate-reducing and non-sulfur-oxidizing bacteria tested. These bacteria are generally considered to be only minor components of the bacterial communities in saline environments (12, 13, 15).

Then, we determined whether the newly designed PCR primers can be applied to natural environments. We used samples of anoxic water from the meromictic Lake Suigetsu, characterized by a permanent large of oxycline at a depth of between 3 and 8 m separating the aerobic freshwater epilimnion from the anaerobic, saline, sulfidogenic hypolimnion (10), since both sulfate-reducing bacteria and phototrophic sulfur bacteria were present in the anaerobic layers of the

lake (13, 14, 17, 23). To provide final confirmation of the specificity of the primers, we sequenced 79 clones (38 clones from a depth of 7 m and 41 clones from 10 m) derived from anoxic water samples of Lake Suigetsu amplified using the primers. All were closely related to the predicted *dsrA* of either green or purple sulfur bacteria based upon a FASTA search (25) of the DNA databases and phylogenetic analysis (Fig. 2). These results indicate that PCR using the *dsrA*-specific primers is useful for the detection of phototrophic sulfur bacteria in environmental samples.

Phototrophic sulfur bacteria are paraphyletic with members belonging to the *Gammaproteobacteria* and the phylum *Chlorobi* and, as a consequence, it is not possible to investi-

Fig. 2. Phylogenetic tree showing the relationships of the *dsrA* clones retrieved from anoxic water of Lake Suigetsu with the *dsrA* from characterized anoxygenic phototrophic sulfur bacteria and sulfate-reducing prokaryotes. Environmental clones are shown in boldface. The sequence is prefixed with the depth of Lake Suigetsu at which the sample was collected on May 2008. The prefix is followed by the clone number. Numbers on the branches represent percentage of 1,000 bootstrap repetitions. Confidence limits of less than 50% are not shown. The distance scale indicates the expected number of changes per sequence position.

gate their ecology by targeting the 16S rRNA gene with a single set of primers or probes. An alternative is to target a functional gene that is characteristic of the group for amplification (1, 3, 8, 28, 34).

Alternative approaches using functional genes that encode APS reductase (*aprBA*), a component of the periplasmic thiosulfate-oxidizing Sox enzyme complex (*soxB*), the M subunit of the photosynthetic reaction center (*pufM*), and the Fenna-Matthews-Olson (FMO) protein (*fmoA*) have been developed to assess the ecology of sulfur-oxidizing microorganisms in natural environments (1, 2, 20–22). However, the phylogenetic relationships of *aprBA* (20) and *soxB* (22) among sulfate-reducing, sulfur-oxidizing prokaryotes, and phototrophic sulfur bacteria make it difficult or impossible to detect both purple and green sulfur bacteria by a single PCR primer set. Furthermore, some phototrophic sulfur bacteria lack either or both of these genes (20, 22). PCR primers that target *pufM* amplify genes in phototrophic non-sulfur bacteria such as *Rhodobacter* and *Rhodomicrobium* as well as purple sulfur bacteria (1, 24). The *fmoA* gene has been used for the specific detection of green sulfur bacteria (2) but not purple sulfur bacteria.

In comparison to PCR assays that target 16S rRNA and other functional genes, our *dsrA*-specific PCR primers appear to amplify from almost all green and purple sulfur bacteria simultaneously. However, it was not possible to design a primer pair that completely excludes all sulfate-reducing prokaryotes. Some sulfate-reducing bacteria can be amplified by our PCR primers; nevertheless, sulfate-reducing bacterial *dsrA* genes were not detected in our clone library analysis. These results show our procedure is useful for ecological studies of phototrophic sulfur-oxidizing microorganisms in the environment.

Phylogenetic analysis

As expected, a ca. 490-bp gene product was obtained from anoxic water samples using the primer set PGdsrAF and PGdsrAR. Clone libraries were made from the *dsrA* PCR products from water samples at 7 and 10 m in May 2008 to identify the various phototrophic sulfur bacteria in Lake Suigetsu. A total of 79 clones were sequenced. Using a >88% similarity cut-off value, the coverage for each library was 92 and 98%, and a rarefaction analysis (Fig. 3) indicated that the

libraries were reasonably well sampled for diversity (6).

To obtain an accurate description of the phylogenetic relationship of the phototrophic sulfur bacterial population in the anoxic water of Lake Suigetsu, we included in our analysis the most characterized *dsrA* sequences of pure phototrophic sulfur bacterial cultures determined in this study and available from sequence databases, as well as from sulfate-reducing bacteria.

A phylogenetic analysis revealed the presence of five lineages of cloned *dsrA* sequences designated as clusters A to E (Fig. 2). The largest cluster of clone sequences grouped in cluster A. These sequences were most closely related to *Chlorobium phaeovibrioides*, a member of the phylum *Chlorobi*. Only two clones related to *C. luteolum* (cluster B) were detected from both depths. The second most abundant group, cluster D, comprises 18 sequences from the anoxic water of Lake Suigetsu. This cluster contains *Chlorobaculum tepidum* TLS and *Chlorobium limicola* DSM 257. The cloned sequence in cluster C was phylogenetically distant from any isolated phototrophic sulfur bacteria but was related to *dsrA* from the green sulfur bacteria. Thus, the sequences in clusters A to D were related to members of the phylum *Chlorobi*, most of which are related to the genera *Pelodictyon* and *Chlorobium*. Green sulfur bacteria of *Prostecochloris*, *Pelodictyon*, and *Chlorobium* have been detected previously mainly in or just below the oxycline of Lake Suigetsu by denaturing gradient gel electrophoresis (DGGE) (17). A recent study found bacteriochlorophylls *e* and *d* in the water in and around the oxycline of Lake Suigetsu (23). These results were consistent with our results in the clone library analysis of *dsrA*. These results suggest green sulfur oxidizers from the genera *Pelodictyon* and *Chlorobium* to be key components of the sulfur and carbon cycles in Lake Suigetsu.

However, only a single purple sulfur bacterial clone, from the family *Chromatiaceae*, was detected from a water sample of Lake Suigetsu. This clone sequence is closely related to *Halochromatium salexigens* DSM 4395^T. Purple sulfur bacteria were occasionally detected at the chemocline of Lake Kaiike, a small saline meromictic lake in Japan similar to Lake Suigetsu (8), while no purple sulfur bacteria were detected in Lake Suigetsu either by DGGE (17) or in photopigment analyses (23). Sequencing of clones derived from our phototrophic sulfur bacterial *dsrA*-specific PCR, however, showed the presence of purple sulfur bacteria in the waters of Lake Suigetsu. This suggests that our PCR procedure can detect minor fractions of microbial communities, which can not be detected by DGGE and chloropigment analyses.

Concluding remarks

The PCR technique described here is a rapid and simple method for the specific detection of phototrophic sulfur bacteria in natural environments that can be readily applied to large numbers of samples. Bacterial cell collection by filtration, DNA extraction, PCR amplification, and agarose gel electrophoresis can be carried out within a day. The procedure should prove a powerful tool for the specific detection and community analysis of phototrophic sulfur bacteria in natural environments. It was applied to water

Fig. 3. Rarefaction curves for *dsrA* in clone libraries from samples collected at 7 m (filled symbols) and 10 m (open symbols) in Lake Suigetsu on 21st May 2008. Bars indicate 95% confidence intervals.

samples from the meromictic Lake Suigetsu. Sequencing of cloned PCR products gave more insight into the existence of different phototrophic sulfur bacterial groups in the anoxic waters. The green sulfur oxidizers, *Pelodictyon* and *Chlorobium*, appear to be important to the sulfur and carbon cycles in Lake Suigetsu, because clones of green sulfur bacteria accounted for a great majority of each library. However, there are still problems to be solved such as the relationship between PCR amplification and cell abundance and the efficiency of the extraction of DNA from bacterial cells. Therefore, it will be necessary to check the specificity for unknown bacteria by sequencing PCR products retrieved from various environments. Further studies on the ecology of the phototrophic sulfur bacteria should be made using real-time PCR to count cell numbers without culture-dependent techniques such as colony counting and Most Probable Number methods.

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