APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 2004, p. 6542–6550 Vol. 70, No. 11
0099-2240/04/$08.00+0 DOI: 10.1128/AEM.70.11.6542–6550.2004

Representative Freshwater Bacterioplankton Isolated from Crater Lake, Oregon

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Received 27 April 2004/Accepted 4 July 2004

High-throughput culturing (HTC) methods that rely on dilution to extinction in very-low-nutrient media were used to obtain bacterial isolates from Crater Lake, Oregon. 16S rRNA sequence determination and phylogenetic reconstruction were used to determine the potential ecological significance of isolated bacteria, both in Crater Lake and globally. Fifty-five Crater Lake isolates yielded 16 different 16S rRNA gene sequences. Thirty of 55 (55%) Crater Lake isolates had 16S rRNA gene sequences with 97% or greater similarity to sequences recovered previously from Crater Lake 16S rRNA gene clone libraries. Furthermore, 36 of 55 (65%) Crater Lake isolates were found to be members of widely distributed freshwater groups. These results confirm that HTC is a significant improvement over traditional isolation techniques that tend to enrich for microorganisms that do not predominate in their environment and rarely correlate with 16S rRNA gene clone library sequences. Although all isolates were obtained under dark, heterotrophic growth conditions, 2 of the 16 different groups showed evidence of photosynthetic capability as assessed by the presence of *puf* operon sequences, suggesting that photoheterotrophy may be a significant process in this oligotrophic, freshwater habitat.

The use of rRNA-based molecular techniques has revolutionized the field of environmental microbiology in recent decades. Studies of microbial diversity are now dominated by analyses of environmental 16S rRNA by gene cloning, sequencing, and in situ hybridization. As a result of these studies, numerous abundant but uncultured microorganisms have been discovered (11, 12, 32, 41, 44, 53). Phylogenetic reconstruction based on 16S rRNA gene sequences has proven to be a powerful tool for organizing and understanding microbial diversity and has revealed many habitat-specific phylogenetic clusters. In many cases, few or no cultured representatives of some of the most numerically dominant phylogenetic clusters are available (18, 19). In particular, Zwart et al. (51) and Glöckner et al. (12) have identified phylogenetic clusters of freshwater bacteria that are frequently found by cloning and sequencing 16S rRNA genes found in lakes around the world. Urbach et al. (44) identified frequently occurring bacterioplankton groups present in Crater Lake. Many of these bacterial groups remain uncultivated. An important goal of environmental microbiology is to understand the ecological roles of the most frequently occurring habitat-specific phylogenetic clusters. To do this, representative organisms from dominant phylogenetic clusters must be identified and cultivated. Studies that link microbial metabolism to environmental variables and biogeochemical cycles may then be initiated.

Crater Lake, located at the crest of the Cascade Mountains in south-central Oregon, was chosen as a microbial ecology study site. This closed-basin, ultraoligotrophic lake receives little allochthonous or anthropogenic nutrient input. Commercial development and recreational activities near the lake are severely restricted by the National Park Service (NPS) to guard its pristine status. It is the deepest lake (589 m) in the United States and is remarkably clear, with primary productivity limited by nitrogen and trace metal availability. An ongoing NPS commitment to research at Crater Lake has resulted in a wealth of readily available limnological data (24, 25, 27, 28). A detailed description of Crater Lake's bacterioplankton community based on 16S rRNA gene cloning and in situ hybridization was published in 2001 (44). This report indicated that the lake bacterioplankton was highly diverse and included many abundant but as yet uncultivated microorganisms. Thus, we set out to isolate and characterize bacterioplankton representative of typical, abundant phylotypes in Crater Lake.

Recent reports indicating that bacterial photoheterotrophy is surprisingly common in oligotrophic marine waters (2, 22, 23) prompted us to screen Crater Lake isolates for the presence of *puf* operon sequences. The *puf* operon is an approximately 40-kb DNA element that contains genes necessary for synthesis of an anoxygenic photosynthetic reaction center (2, 33). This operon has a remarkable tendency to transfer horizontally between different groups of bacteria (30, 33). Its frequent occurrence in a wide diversity bacterioplankton species in marine oligotrophic environments suggests that selective forces may favor its acquisition in low-nutrient habitats (2). The ultraoligotrophic status of Crater Lake is likely to favor the survival of organisms able to switch between heterotrophy and phototrophy as light availability and organic carbon levels fluctuate. Facultative photoheterotrophy may play an important role in primary productivity and in the maintenance of low levels of dissolved organic carbon in freshwater oligotrophic environments, similar to its speculated role in marine oligotrophic environments.

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MATERIALS AND METHODS

Cultures. Samples of Crater Lake water were obtained aboard the NPS research vessel Neuston moored over the deepest part of the lake. Niskin contain-

ers were deployed at 15, 130, 300, and 500 m to obtain bacterial isolation samples and at 300 m to obtain natural water as a base to prepare culture media. Samples for bacterial isolation were kept at 4°C and inoculated into culture medium within 72 h of collection. Acid-washed polycarbonate containers were used for water, live samples, and medium preparation whenever possible. Crater Lake water to be used for medium was filtered through a 0.2-μm-pore-size membrane filter and immediately autoclaved. To prepare media, sterile, concentrated stocks of nutrients were added to sterile Crater Lake water. Mixed carbon medium consisted of 0.001% D-glucose, 0.001% D-ribose, 0.001% pyruvate, 0.001% succinate, 0.001% glycerol, 0.001% *N*-acetylglucosamine, 0.002% ethanol, 1 μM NH_4Cl, 0.1 μM $K_2HPO_4 \cdot 3H_2O$, 100 μg of inositol/liter, 200 μg of thiamine HCl/liter, 0.1 μg of cyanocobalamin/liter, 0.1 μg of biotin/liter, 0.2 μg of folic acid/liter, 1 μg of *p*-aminobenzoic acid/liter, 10 μg of niacin/liter, 20 μg of calcium pantothenate/liter, and 10 μg of pyridoxine/liter. Yeast extract-acetate medium consisted of 0.001% yeast extract, 0.001% sodium acetate, 325 μM $(NH_4)_2SO_4$, 20 μM KCl, 10 μM K_2HPO_4, 7 μM $MgSO_4 \cdot 7H_2O$, and 0.6 μM $Ca(NO_3)_2 \cdot 4H_2O$. To determine bacterioplankton densities for inoculation, direct cell counts were done by collecting 5-ml samples of Crater Lake water onto 13-mm-diameter, 0.2-μm-pore-size polycarbonate filters. The filters were fixed with 1% formaldehyde and stained for 5 min with 5 μg of DAPI (4′,6′-diamidino-2-phenylindole)/ml. Membrane filters were mounted on oiled slides and viewed by fluorescence microscopy. Forty-eight-well culture plates were inoculated with 25 or 50 cells per well in a 1-ml volume of medium. Plates were incubated in the dark at 4, 10, or 15°C for 3 to 22 weeks. To detect cell growth, 48-well plate cell culture arrays were prepared, DAPI stained, and observed by fluorescence microscopy as previously described (7). Percent culturability was determined by the equation for estimation of culturability, $V = -\ln(1 - p)/X$, as described by Button et al. (6), where V is the estimated culturability, p is the proportion of wells positive for growth (wells positive for growth/total inoculated wells), and X is the initial inoculum of cells added per well.

Wells positive for growth were identified, and cultures were transferred to 10 ml of fresh medium for 3 to 6 weeks of additional incubation. Ten-milliliter cultures were screened with cell array technology, and genomic DNA was isolated from positive cultures demonstrating a single morphological type. For long-term storage, glycerol was added to cultures to a final concentration of 10%, and the cells were frozen at −70°C. A representative from each of the 16 different isolates was recoverable from the frozen state and verified morphologically. To determine culturability by traditional methods, inocula of 20 μl of viable culture were applied to spread plates of a 0.1 dilution of R2A (10% R2A) (34).

Imaging. Two hundred microliters of bacterial culture was fixed and stained with 1% formaldehyde and 5 μg of DAPI/ml followed by filtration onto 0.2-μm-pore-size polycarbonate filters as described by Connon and Giovannoni (7). Filters were mounted on oiled microscope slides and viewed at ×1,000 magnification with a Leica DMLS epifluorescence microscope. Digital images were acquired with a Spot Jr. camera (Diagnostic Instruments, Inc.). Size measurements were made using Spot imaging software version 3.3.2.

DNA sequencing. To obtain bacterial pellets for DNA extraction, 10 ml of culture was centrifuged at 2,900 × *g* for 30 min. Genomic DNA was extracted from bacterial pellets by using a DNeasy tissue kit (QIAGEN) and the manufacturer's protocol for gram-positive bacteria. 16S rRNA gene sequences were amplified from genomic DNA by PCR using primers 27F (5′-AGR GTT YGA TYM TGG CTC AG-3′) and 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′). *puf* operon sequences were amplified with the primers *puf*L-F (5′-CTK TTC GAC TTC TGG GTS GG-3′) and *puf*M-R (5′-CCA TSG TCC AGC GCC AGA A-3′). The PCRs contained 0.025 U of *Taq*/μl, 5% acetamide, 1.5 mM $MgCl_2$, a 200 nM concentration of each primer, 220 μM (each) deoxynucleoside triphosphates, 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, and 0.01% Tween 20. PCR conditions were 35 cycles of 94°C denaturation for 30 s, 55°C (16S rRNA amplification) or 53°C (*puf* operon amplification) annealing for 1 min, and 72°C extension for 2 min, with a final 7-min extension. PCR products were purified using Qiaquick (QIAGEN) kit reagents. Purified PCR products were sequenced by using BigDye3 chemistry and an ABI genetic analyzer 310 (Applied Biosystems, Foster City, Calif.) according to the manufacturer's instructions. 16S rRNA sequencing primers were 27F, 1492R, 519F (5′-CAG CMG CCG CGG TAA TWC-3′), 519R (5′-GWA TTA CCG CGG CKG CTG-3′), 926F (5′-AAA CTY AAA KGA ATT GAC GG-3′), and 926R (5′-CCG TCA ATT CMT TTR AGT TT-3′). *puf* operon sequencing primers were *puf*L-F and *puf*M-R (1).

16S rDNA phylogenetic analysis. 16S rDNA sequences were added to the ARB rRNA sequence database Cluster_AEM_all.arb obtained from the public FTP server ftp://ftp.mpi-bremen.de/pub/molecol_p/fog-aem/ (12). This database included a phylogenetic analysis of over 14,000 16S rRNA gene sequences of clones and isolates, many of which were derived from freshwater sources. Automated sequence alignments were verified and corrected manually with consideration of 16S rRNA secondary structure (40). By using the parsimony insertion tool in ARB, Crater Lake high-throughput culture collection (HTCC) sequences (358 to 472 bp in length) were added to a phylogenetic consensus tree previously constructed by Glöckner et al. (12). This approach identified HTCC sequences that were phylogenetically related to globally distributed freshwater clusters. To refine this analysis, longer 16S rRNA gene sequences (>1,000 bp) were obtained for isolates that clustered within or adjacent to the freshwater clusters described by Glöckner et al. Crater Lake HTCC sequences and sequences within each freshwater cluster were used to prepare neighbor-joining trees. A base frequency mask was generated to exclude the most variable positions. The base frequency mask filtered out all alignment positions with less than 50% agreement. Masked alignments were exported to PAUP (42) for bootstrapped neighbor-joining analysis using Jukes-Cantor distance correction and 1,000 replicates. Missing and ambiguous positions were excluded from analysis. *Alphaproteobacteria* analysis included 546 characters, *Betaproteobacteria* analysis included 1,193 characters, and *Cytophaga-Flavobacteria* (CF) analysis included 949 characters. Sequence similarities were calculated from aligned sequences by preparing distance matrices using Jukes-Cantor distance corrections and omitting missing or ambiguous characters.

PufM and PufL phylogenetic analysis. Partial *puf* operon sequences were obtained using 5′ primer *puf*L-F and 3′ primer *puf*M-R. Sequence translation using standard genetic code from primer *puf*L-F yielded a partial PufL protein sequence, and sequence translation from primer *puf*M-R yielded a partial PufM protein sequence. Related PufL and PufM amino acid sequences were downloaded from GenBank for use in phylogenetic analysis. Puf protein sequences were aligned with Pileup in Wisconsin Package version 10.2 from Genetics Computer Group, Madison, Wis.; verified manually; and exported to PAUP. Bootstrapped neighbor-joining trees were constructed using distance, minimum evolution model, and 1,000 replicates. Missing and ambiguous bases were excluded from analysis. PufL analysis included 149 amino acid characters; PufM analysis included 171 amino acid characters. Sequence similarity values were obtained from distance matrices generated by PAUP.

Nucleotide sequence accession numbers. HTCC 16S rRNA gene sequences are deposited in GenBank under accession numbers AY584571 to AY584586. Puf operon sequences are deposited under accession numbers AY584587 (HTCC515 *pufL*), AY584588 (HTCC528 *pufL*), and AY584589 (HTCC *pufM*).

RESULTS

Isolation, culturability, and morphological data. Crater Lake is known for its extreme clarity and typically has deep chlorophyll maxima (25). Maximum chlorophyll concentration was observed at 130 m on the day of sampling. Bacterioplankton concentrations at 15, 130, 300, and 500 m were determined to be 2.4×10^5, 3.5×10^5, 1.3×10^5, and 0.7×10^5 cells/ml, respectively. Lake temperature below 100 m is approximately 3.5°C year-round and from 4 to 17°C at the surface, and dissolved oxygen is present throughout the water column (25, 28, 44). In an attempt to mimic natural conditions, cultures were initiated aerobically at 4, 10, and 15°C in the dark. Key features of the high-throughput culturing (HTC) method used in this study include culture media consisting of sterilized natural water amended with very low levels of organic and mineral nutrients and extinction culturing in liquid media to obtain pure cultures (7, 37). Isolates cultured in mixed carbon or yeast extract-acetate media were obtained from lake depths of 15, 130, 300, and 500 m, and details are summarized in Table 1. One hundred fifty-three of 672 extinction culture wells were positive for bacterial growth. Culturability ranged from 0.1 to 2.6%, with an overall average of 0.8%. This low rate of culturability may reflect an intrinsic inability of highly diverse populations to grow in a single medium, a high frequency of dormant or noncultivable cells, nutrient limitations in the media used, or inhibitory culture conditions. Organisms collected from 15 and 130 m had an average culturability of 1.8%,

TABLE 1. Classification and isolation and culture characteristics of 55 Crater Lake isolates in 16 groups

Isolate HTCC no(s).	Division	No. of isolates at specific depths (m)				Growth in 10% R2A	Morphology and dimensions
		15	130	300	500		
500–502	*Alphaproteobacteria*			2	1	−	Rod, 0.5 × 1.0 μm
503–513	*Alphaproteobacteria*	8			3	−	Rod, 0.5 × 0.8 μm
515–517	*Alphaproteobacteria*	3				−	Rod, 0.5 × 1.5 μm
518–524	*Betaproteobacteria*		7			+	Rod, 0.5 × 2.5 μm
525–527	*Betaproteobacteria*				3	−	Vibrio, 0.6 × 3.1 μm
528–539	*Betaproteobacteria*	1	5	5	1	−	Rod, 0.7 × 3.5 μm
540	*Betaproteobacteria*			1		−	Rod, 0.5 × 5.0 μm
541–542	*Betaproteobacteria*			1	1	−	Rod, 0.7 × 8.1 μm
543	*Betaproteobacteria*		1			−	Coccus, 0.5-μm diameter
544–549	*Gammaproteobacteria*		5	1		+	Rod, 0.7 × 1.0 μm
550	*Gammaproteobacteria*				1	+	Rod, 0.5 × 1.1 μm
551	*Gammaproteobacteria*			1		−	Coccobacillus, 0.5 × 0.7 μm
552	*Bacteroidetes*	1				+	Rod, 0.7 × 4.7 μm
553	*Bacteroidetes*	1				+	Rod, 0.7 × 4.4 μm
554	*Bacteroidetes*			1		−	Filament, 0.5 × 8.5 μm
555	*Actinobacteria*				1	+	Rod, 0.5 × 1.0 μm

whereas organisms from 300 and 500 m had an average culturability of 0.6%.

Several of the 153 positive extinction cultures were transferred to 10 ml of culture for further growth and characterization. Fifty-five independent isolates were characterized morphologically and by 16S rRNA gene sequencing. These isolates were named HTCC500 through HTCC555, excluding HTCC514, an isolate that was not maintained as a pure culture. DNA sequencing revealed that the 55 isolates were represented by 16 different 16S rRNA gene sequences, grouped as shown in Table 1.

Because HTC culturability was similar to that obtained by more-traditional isolation techniques (5), it seemed plausible that similar isolates could have been obtained more simply by plating on relatively nutrient-rich agar. One isolate from each group was plated on 10% R2A and evaluated for colony formation. As shown in Table 1, only 6 of 16 unique isolates were able to form colonies on 10% R2A. The highly nutrient-limited status of Crater Lake has likely resulted in selection for obligately oligotrophic microorganisms that fail to grow or form colonies in traditional, nutrient-rich isolation media. Hahn et al. have shown that some freshwater isolates that fail to grow initially in nutrient-rich media may be gradually acclimated to rich media (15). Our technique of extinction culturing in liquid, low-nutrient medium does not require any rich medium acclimatization or colony formation. A representative culture from each of the 16 different types of isolates was DAPI stained and photographed as shown in Fig. 1. Cellular morphologies and sizes are summarized in Table 1.

Sequence similarity analysis and comparison to Crater Lake clone library sequences. A BLAST analysis of Crater Lake isolate 16S rRNA gene sequences revealed many close matches to unidentified bacterial 16S rRNA gene clones in GenBank and matches to previously described isolates. Table 2 shows the names and degrees of sequence relatedness of the described microorganisms closest to Crater Lake isolates. Based on the rule of thumb that a bacterium can be considered a novel species if its 16S rRNA gene sequence has less than 97% similarity to any described species (35, 47), it appears that HTCC515 to HTCC517, HTCC525 to HTCC527, HTCC540, and HTCC551 are novel species. Crater Lake 16S rRNA gene clone library sequences (44) were recovered from GenBank and aligned to isolate sequences. As shown in Table 2, 30 of 55 (55%) HTCC Crater Lake isolates had 16S rRNA sequences greater than 97% similar to sequences present in Crater Lake 16S rRNA gene clone libraries. Sequences from Crater Lake clone libraries were previously analyzed and organized into 37 phylogenetic clusters. While the majority of clone sequences grouped with *Actinomycetes*, *Cyanobacteria*, and *Verrucomicrobiales*, significant populations of *Alphaproteobacteria*, *Betaproteobacteria*, and *Chlorobiaceae* were also identified (44). Table 3 shows the affiliation of Crater Lake isolates with Crater Lake phylogenetic clusters previously identified by 16S rRNA gene clone library sequence analysis (44). Fifteen Crater Lake isolates (HTCC528 to HTCC539, HTCC540, and HTCC541 to HTCC542) group within the *Comamonadaceae* phylogenetic cluster, a group of bacteria found in Crater Lake and in lakes around the world. The high degree of correlation between isolate 16S rRNA gene sequences and Crater Lake clone library sequences suggests that the isolation method used effectively recovered representatives of phylogenetic groups previously found to be abundant in Crater Lake.

Affiliation of Crater Lake isolates with globally distributed bacterioplankton groups. 16S rRNA gene sequences from Crater Lake isolates were added to an ARB database including over 14,000 16S rRNA gene sequences initially prepared by Glöckner et al. (12) and expanded to include recent freshwater sequences deposited in GenBank. Crater Lake isolate sequences were aligned to their closest relatives in the database, and preliminary phylogenetic analyses were performed. Data sets were exported to PAUP (42) for preparation of bootstrap consensus neighbor-joining trees. Figure 2 shows the phylogenetic relatedness of Crater Lake isolate sequences to members of globally distributed freshwater clusters previously described by Glöckner et al. (12) and Zwart et al. (51).

Alphaproteobacteria isolated from Crater Lake included 17 isolates, numbered HTCC500 to HTCC517. HTCC503 to HTCC513 grouped within cluster GOBB3-C201 and adjacent to cluster alpha IV. HTCC500 to HTCC502 are closely related to HTCC503 to HTCC513 but do not fall within cluster

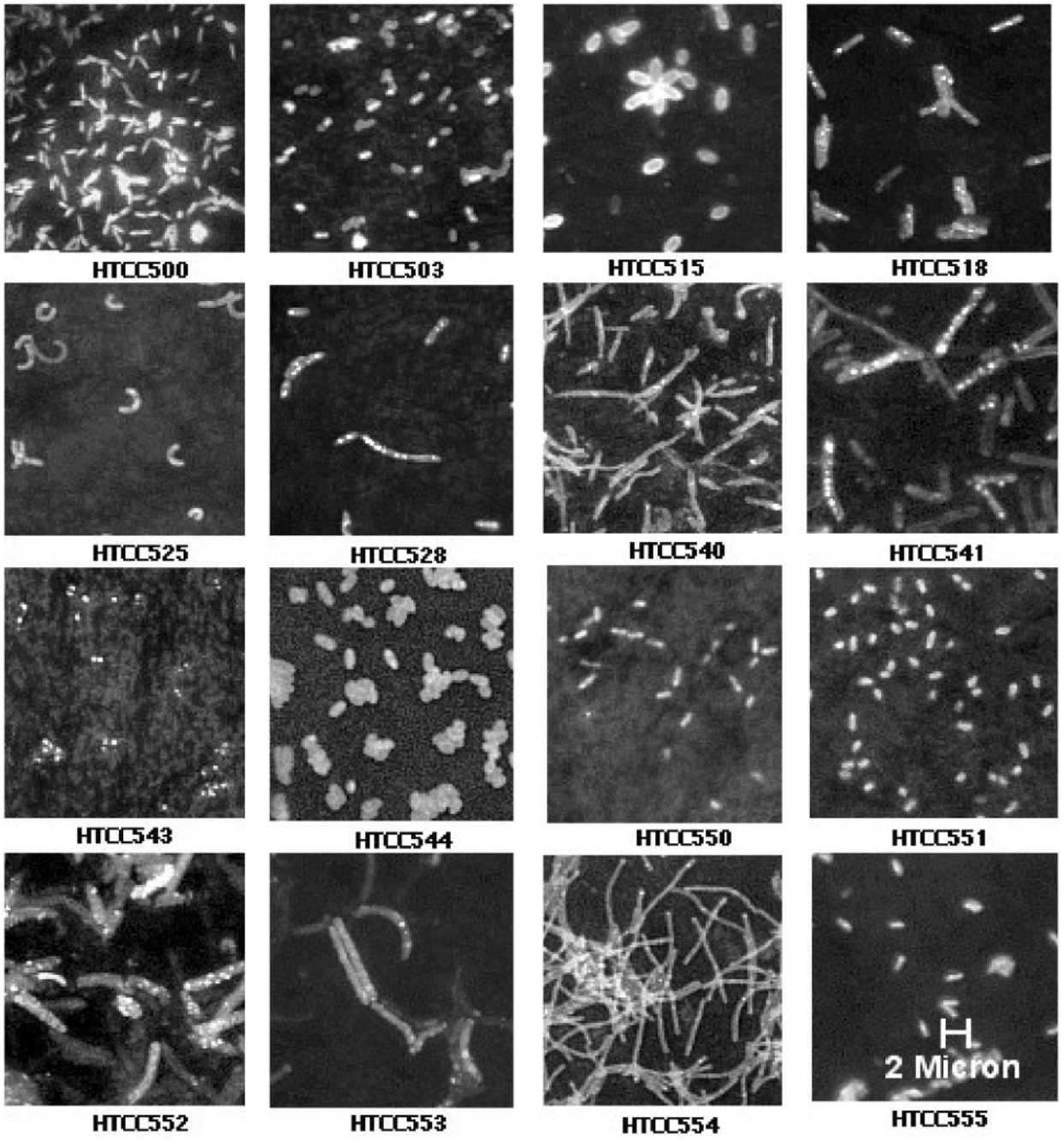

FIG. 1. Photographic images of DAPI-stained Crater Lake isolates. Scale bar in lower right panel applies to all panels.

GOBB3-C201 because their sequence similarity to cluster members is less than 99%. HTCC515 to HTCC517 are members of the Crater Lake *Rhodobacter* cluster described by Urbach et al. (44) but are not members of any cluster described by Zwart et al. (51, 53) or Glöckner et al. (12).

Betaproteobacteria HTCC528 to HTCC539, HTCC540, and HTCC541 to HTCC542 all grouped within the beta I cluster, with HTCC528 to HTCC539 additionally affiliated within the GKS16 cluster. Until now, no cultivated species belonging to cluster GKS16 have been described in a scientific publication, although several GKS16-related 16S rRNA gene sequences from bacteria cultured from glacial ice are present in GenBank. Based on 16S rRNA gene sequence similarity, the closest cultivated species to HTCC528 that is described in a publication is *Polaromonas vacuolata* (21). Beta I cluster species dominate in many lakes (12, 17, 32, 52) and include species in the genera *Rhodoferax* and *Variovorax* (10, 16).

The *Betaproteobacteria* HTCC543 16S rRNA gene sequence is 99% identical to that from *Polynucleobacter necessarius*, an obligate endosymbiont of the freshwater protozoan *Euplotes aediculatus* (36, 39). 16S rRNA gene clone libraries from lakes around the world frequently include sequences closely related

TABLE 2. Relatedness of Crater Lake isolates to known organisms, Crater Lake clone library sequences, and previously described freshwater clusters

Isolate HTCC no(s). (GenBank accession no.)	Nearest described relative (GenBank accession no.) (reference)	Sequence similarity (%)	Crater Lake clone relatives	Freshwater cluster affiliations
500–502 (AY584571)	*Sphingomonas* sp. strain B18 (AF410927) (49)	97	CL0–16, -60, -92, CL500–33	*Sphingomonas*[a]
503–513 (AY584572)	*Sphingomonas* sp. strain B18 (AF410927) (49)	98	CL0–16, -60, -92, CL500–33	*Sphingomonas*[a] GOBB3-C201[c]
515–517 (AY584573)	*Rhodobacter sphaeroides* (X53855) (20)	95	CL0–56, -89	*Rhodobacter*
518–524 (AY584574)	*Iodobacter fluviatilis* (AJ247214) (29)	99		
525–527 (AY584575)	*Aquaspirillum autotrophicum* (AB074524) (9)	96		
528–539 (AY584576)	*Polaromonas vacuolata* (U14585) (21)	97	CL0–90, 99, 102, 119, CL500–45, -61	*Comamonadaceae*[a] Beta I[b] GKS16[c]
540 (AY584577)	*Aquaspirillum delicatum* (AF078756) (9)	96		*Comamonadaceae*[a] Beta I[b]
541–542 (AY584578)	Antarctic bacterium R-7724 (AJ440986) (46)	98		*Comamonadaceae*[a] Beta I[b]
543 (AY584579)	Isolate MWH-HuK1 (AJ550665) (14)	99		Beta II[b] *P. necessarius*[c]
544–549 (AY584580)	*Pseudomonas lini* (AY035996) (8)	98		
550 (AY584581)	*Pseudomonas* sp. strain NZ099 (AF388207) (13)	97		
551 (AY584582)	*Pseudomonas* sp. strain NZ017 (AY014805) (13)	96		
552 (AY584583)	*Flectobacillus* isolate EP293 (AF493693) (31)	98	CL0–73, -103	*Flectobacillus*[a] CF III[b] GKS2-216[c]
553 (AY584584)	*Flectobacillus* isolate EP293 (AF493693) (31)	97		*Flectobacillus*[a] CF III[b] GKS2-216[c]
554 (AY584585)	*Flavobacterium limicola* (AB075230) (43)	98		*Flavobacterium*[a] CF II[b]
555 (AY584586)	*Geodermatophilus* sp. strain BC496 (AJ296066) (45)	98		

[a] As described by Urbach et al. (44).
[b] As described by Glöcker et al. (12).
[c] As described by Zwart et al. (51).

to *P. necessarius*, and several free-living isolates were recently obtained from freshwater sources (4, 14). This group of organisms is described as freshwater cluster beta II (12) or as the *P. necessarius* cluster (51).

Bacteroidetes strains HTCC552 and HTCC553 have a 96% 16S rRNA sequence similarity to each other and identical morphologies. As shown in Fig. 2, they align with the CF III freshwater cluster that includes *Flectobacillus*. They also fit within cluster GKS2-216, described by Zwart et al. (51). The 16S rRNA gene sequence of *Bacteroidetes* strain HTCC554 is closely related to that of several different species in the genus *Flavobacterium* and groups within freshwater cluster CF II.

Table 2 summarizes Crater Lake isolate freshwater cluster affiliations. The majority of the isolates are members of freshwater clusters described by Glöckner et al. (12), Urbach et al. (44), or Zwart et al. (51). Thus, HTC effectively recovered bacteria representative of typical lake bacterioplankton, as indicated by their affiliation with previously defined, globally distributed, freshwater clusters.

***puf* operon sequences.** Phylogenetic analysis indicated that some Crater Lake isolates were close relatives of phototrophic bacteria. While all of the isolates were obtained under heterotrophic growth conditions, it is possible that some of them are also capable of photosynthesis, making them photoheterotrophic. To test this hypothesis, 16 different isolates (one representative from each unique 16S rRNA gene sequence) were screened for the presence of a set photosynthesis reaction center genes, the *puf* operon. *puf* operon sequences were amplified by PCR from isolates HTCC515, a *Rhodobacter* relative, and HTCC528, a member of the beta I freshwater cluster related to *Polaromonas* (Table 2) (21). A phylogenetic analysis of PufL and PufM protein sequences is shown in Fig. 3. The 16S rRNA gene sequence of HTCC515 shows 95% sequence similarity to *Rhodobacter sphaeroides*, a purple bacterium capable of anoxygenic photosynthesis (20, 48). The HTCC515 PufL protein sequence clusters phylogenetically with PufL sequences from species in the genus *Rhodobacter* and has the greatest amount of amino acid similarity (77%) to *Rhodobacter sphaeroides* PufL.

Interestingly, PufL sequences from the HTCC isolates did not sort into clades consistent with phylogenetic predictions based on 16S rRNA gene sequences, suggesting horizontal transfer. As shown in Fig. 3, many *Alphaproteobacteria* have PufL sequences that are more similar to *Betaproteobacteria* Puf

TABLE 3. Relatedness of Crater Lake isolates to phylogenetic clusters previously identified in Crater Lake 16S rRNA gene clone libraries (34)

Isolate(s)	Phylogenetic cluster	Percent similarity to library clones	Percent of clone library		
			0 m	120 m	500 m
HTCC500–HTCC513	*Sphingomonas*	95.4–100	3.1	0	4.1
HTCC515–HTCC517	*Rhodobacter*	99.2–99.7	2.1	0	0
HTCC528–HTCC542	*Comamonadaceae*	87.0–98.8	5.2	4.7	6.8
HTCC552–HTCC553	*Flectobacillus*	95.6–99.7	2.1	0	0
HTCC554	*Flavobacterium*	94.4–94.7	3.1	0	0

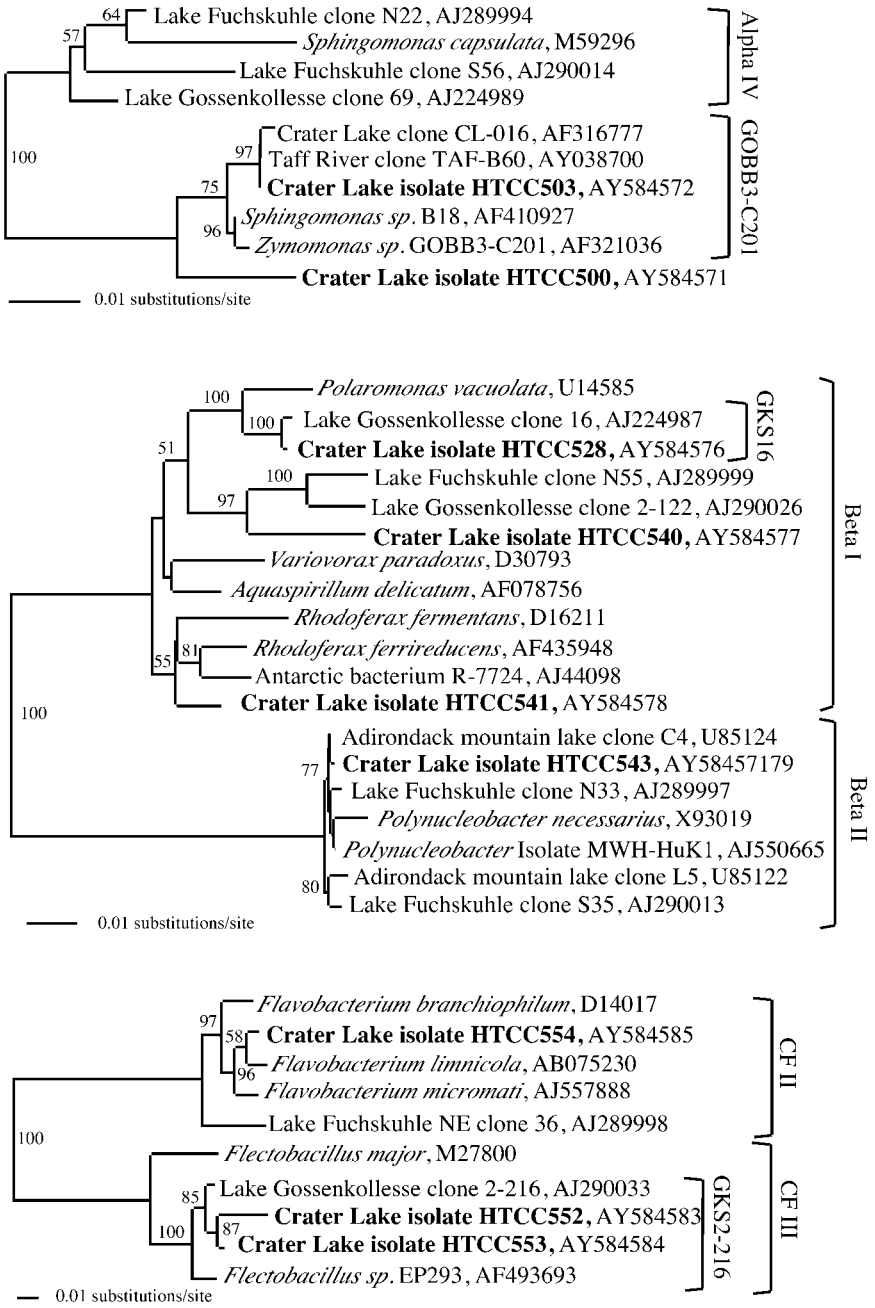

FIG. 2. Phylogenetic relationships between Crater Lake isolates and globally distributed freshwater bacteria based on neighbor-joining 16S rRNA gene sequence analysis. Bootstrap values below 50 are not shown. Freshwater clusters alpha IV, beta I, beta II, CF II, and CF III were defined by Glöckner et al. (12). Freshwater clusters GOBB3-C201, GKS16, and GKS2-216 were defined by Zwart et al. (51).

sequences than to those of other *Alphaproteobacteria*, such as *Rhodobacter*. PufL from Crater Lake isolate HTCC528 showed the greatest amount of amino acid similarity to PufL from the alphaproteobacterium *Rhodospirillum centenum* (77%) but was also very similar to PufL from the betaproteobacteria *Rubrivivax gelatinosus* (76%), *Roseateles depolymerans* (76%), and *Rhodoferax fermentans* (72%). Apparently, use of *puf* operon sequences as a phylogenetic marker may be of limited value due to the tendency of these sequences to transfer horizontally (30, 33).

When PufM protein sequences were analyzed, HTCC528 PufM clustered with other betaproteobacterial PufM sequences, mirroring HTCC528's 16S rRNA-based phylogenetic affiliation with *Betaproteobacteria* (Fig. 3B). HTCC528 is a member of freshwater cluster beta I that is abundant in many lakes. This cluster was initially defined by over 100 closely related 16S rRNA sequences, the vast majority of which were from freshwater clone libraries (12). Five described species are within the cluster, including the known photoheterotroph *Rhodoferax fermentans* (16). The discovery that HTCC528 contains

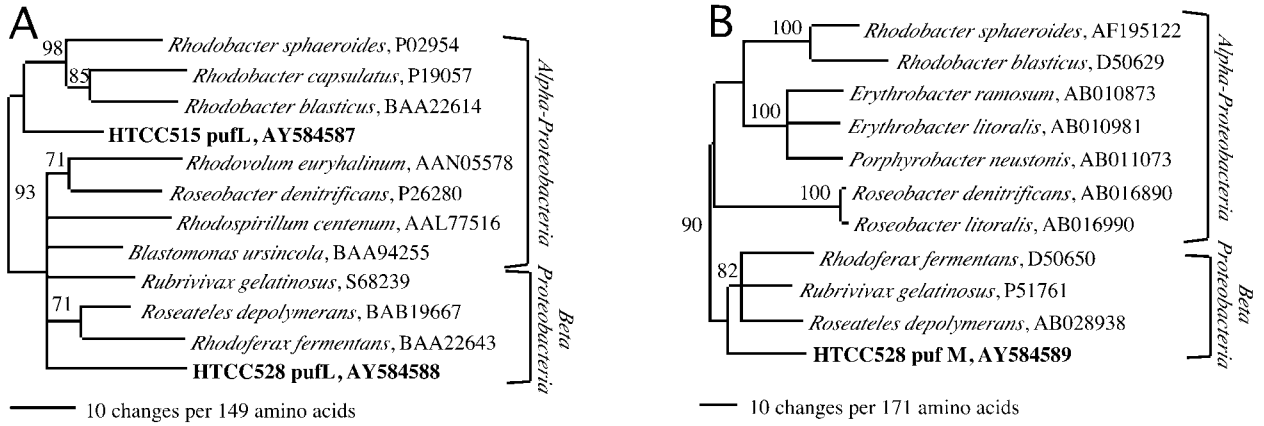

FIG. 3. Neighbor-joining phylogenetic analysis of PufL (A) and PufM (B) amino acid sequences inferred from gene sequences.

puf operon sequences provides evidence that photoheterotrophy may be an important characteristic of beta I freshwater cluster organisms.

DISCUSSION

Isolation and characterization of ecologically important prokaryotes are essential to understanding the vital roles they play in geochemical cycles. We used HTC methods to isolate typical lake bacterioplankton from Crater Lake. 16S rRNA-based phylogenetic reconstructions and comparison of isolate sequences with clone library sequences were used to determine the relationship of each isolate to phylogenetic groups thought to be abundant in lakes. Low-nutrient media were used to obtain the isolates described in this study. The success of this strategy was evidenced by tests that revealed the inability of most of the isolates to grow on 10% R2A, a traditional bacterioplankton isolation medium (34). The liquid, low-nutrient media employed resulted in cultures rich in isolates belonging primarily to *Bacteroidetes* and *Alpha-*, *Beta-*, and *Gammaproteobacteria* divisions. We did not isolate representatives of *Cyanobacteria*, *Actinomycetes*, *Verrucomicrobiales*, green nonsulfur bacteria, *Planctomycetales*, *Crenarchaeota*, or candidate division OP10, even though these groups are represented in rRNA gene clone libraries from Crater Lake (44) and other freshwater environments (12, 53). Recently, Bruns et al. (3) reported the isolation of previously uncultured freshwater actinobacteria from a eutrophic lake when the signal molecule cyclic AMP was added to liquid enrichment culture media. Thus, in some cases, a simple addition to culture medium formulation can dramatically improve culturability. Hahn et al. used stepwise culture acclimatization techniques to enrich ultramicrobacteria classified as actinobacteria (15). No single isolation medium can isolate all of the prokaryotes from a diverse environment, such as Crater Lake. However, it is possible that the application of HTC methods using a series of rationally designed media will eventually yield isolates representing all dominant bacterioplankton groups. Thirty-six of the 55 isolates had 16S rRNA gene sequences that were highly similar to Crater Lake and/or other freshwater 16S rRNA gene clone library sequences, thus validating the HTC method as a way to obtain isolates that are representative of numerically significant clades.

Three different groups of Crater Lake isolates, HTCC528 to HTCC539, HTCC540, and HTCC541 to HTCC542, were found to be members of the beta I freshwater cluster. *puf* operon sequences were PCR amplified from HTCC528 but not HTCC540 or HTCC541, suggesting that HTCC528 has the ability to carry out photosynthesis. The beta I cluster is a very diverse and widely distributed bacterial group that includes both photosynthetic (*Rhodoferax fermentans* and *Variovorax paradoxus*) and nonphotosynthetic (*Hydrogenophaga palleronii*, *Comamonas testosteroni*, and *Brachymonas denitrificans*) bacteria (12). Sequences that hybridize to beta I bacterial probes have been found abundantly associated with macroscopic organic aggregates (lake snow) from Lake Constance, providing further evidence for the heterotrophic role played by at least some beta I bacteria (38). While beta I bacteria are frequently found to be abundant in lakes, relatively few isolates are available. The addition of HTCC528, HTCC540, and HTCC541 to the current set of beta I isolates provides an important resource for elucidation of the ecological roles played by this widespread group of bacterioplankton.

Several different phylogenetic clusters of *Alphaproteobacteria* are commonly found in freshwater environments (12, 44, 46, 52, 53), although their abundance is estimated to be relatively low (11, 44). Crater Lake HTCC isolates included three groups of different *Alphaproteobacteria*. Two of these, HTCC500 to HTCC502 and HTCC503 to HTCC513, were related to several *Sphingomonas* species and freshwater clusters alpha IV and GOBB3-C201. Analysis of 16S rRNA gene and *puf* operon sequences indicated that strains HTCC515 to HTCC517 are examples of a novel *Alphaproteobacteria* species related to *Rhodobacter*, a genus of photoheterotrophic purple bacteria (50).

Based on 16S rRNA gene sequences, three different Crater Lake HTCC isolates were classified in the division *Bacteroidetes* and were found to be members of the freshwater CF II or CF III and GKS2-216 clusters. HTCC552 and HTCC553 were closely related to *Flectobacillus* sp. strain EP293 (31), and HTCC554 was closely related to several *Flavobacterium* species. Several isolates from these genera are already available. Crater Lake clone library analysis indicated that organisms in the division *Actinobacteria* were abundant in Crater Lake. A single HTCC isolate, HTCC555, was classified in this division. The HTCC555 16S rRNA gene sequence was highly similar to

that from species within the genus *Geodermatophilus* and does not appear to be represented in Crater Lake or other lake clone libraries and is not a member of any previously defined freshwater cluster. Three different HTCC isolates were classified as *Gammaproteobacteria* closely related to *Pseudomonas*, a genus known for metabolic flexibility and tendency to be selected for in heterotrophic enrichment cultures but not numerically dominant in freshwater.

The availability of a set of isolates that represent typical bacterioplankton from an oligotrophic environment provides a resource for experiments aimed at revealing the metabolic activities of frequently occurring microbial groups. For example, we probed our set of heterotrophic Crater Lake isolates for the presence of genes involved in anoxygenic photosynthesis. Two of the 16 different groups, representing 15 of 56 independent isolates, contained *puf* operon sequences (HTCC515 to HTCC517 and HTCC528 to HTCC539). Interestingly, these isolates are very closely related to eight different Crater Lake 16S rRNA gene clones (Table 2) (44). This result suggests that photosynthetic capabilities may be surprisingly common in freshwater bacterial oligotrophs. Kolber and colleagues published the first report showing that anoxygenic bacterial photosynthesis could be an important process in the oceans (23). Subsequently, Beja and collaborators showed that the photosynthetic reaction center genes needed for anoxygenic bacterial photosynthesis are distributed among diverse taxa of uncultivated marine proteobacteria (2). Anoxygenic photosynthesis by a diverse set of purple bacteria is also believed to contribute significantly to the primary productivity of a permanently frozen Antarctic lake (22). The results we report here indicate that anoxygenic photosynthesis may be important in temperate, oligotrophic freshwater as well as marine oligotrophic ecosystems. The *puf* operon contains several genes needed for synthesis of a photosynthetic reaction center. Bacterial operon structure may be preserved by evolutionary forces that keep physiologically related genes arranged contiguously, such that a single horizontal DNA transfer event can carry all the information necessary to confer a complex metabolic function, such as light harvesting to capture energy (26, 33). Here we present evidence that PufL sequence phylogeny is not congruent with 16S rRNA gene phylogeny, an observation consistent with the notion that the *puf* operon tends to transfer horizontally (30, 33).

Crater Lake is a deep, nitrogen-limited lake with a relatively low density of bacterioplankton (25, 27, 44). Its ultraoligotrophic status has likely selected for obligately oligotrophic organisms and organisms able to switch between heterotrophy and phototrophy as light availability and organic carbon levels fluctuate. During summer and fall, chlorophyll concentrations peak at approximately 150 m, and 20 to 25% of total chlorophyll partitions with picoplankton, presumably bacterioplankton, or very small cyanobacteria in the 0.22- to 2.0-μm size range (24). The contribution made by (noncyanobacterial) bacterioplankton to the primary productivity of Crater Lake remains a tantalizing question. The discovery that two different groups of heterotrophic Crater Lake isolates contained *puf* operon sequences suggests that facultative photoheterotrophy may play a role in primary productivity and in the maintenance of low levels of dissolved organic carbon in freshwater oligotrophic environments.

ACKNOWLEDGMENTS

We are indebted to Mark Buktenica of NPS for help with sample collection at Crater Lake National Park, Bob Collier for chlorophyll data, Kevin Vergin for technical assistance, and Gary Larson, Jang Cho, Carol DiMeo, and Mike Rappe for useful discussions.

This research was supported by National Science Foundation grants MCB-0237713 to S.J.G. and MRI 0115892 to K.A.P. and by travel funds provided by Southern Oregon University.

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