

Remarkable Diversity of Phototrophic Purple Bacteria in a Permanently Frozen Antarctic Lake

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Although anoxygenic photosynthesis is thought to play an important role in the primary productivity of permanently frozen lakes in the Antarctic dry valleys, the bacterial communities responsible for this metabolism remain uncharacterized. Here we report the composition and activity of phototrophic purple bacteria in Lake Fryxell, Antarctica, as determined by analysis of a photosynthesis-specific gene, *pufM*. The results revealed an extensive diversity and highly stratified distribution of purple nonsulfur bacteria in Lake Fryxell and showed which phylotypes produced *pufM* transcripts in situ. Enrichment cultures for purple bacteria yielded two morphotypes, each with a *pufM* signature identical to signatures detected by environmental screening. The isolates also contained gas vesicles, buoyancy structures previously unknown in purple nonsulfur bacteria, that may be necessary for these organisms to position themselves at specific depths within the nearly freezing water column.

Lake Fryxell is a meromictic lake located 18 m above sea level at the entrance of Taylor Valley adjacent to McMurdo Sound, Antarctica (17). The lake is a closed basin with a maximum depth of 19 m (28). Lake Fryxell has undergone several dry-down periods in the past, resulting in slightly saline bottom waters overlaid with more dilute water introduced from glacial meltwater streams (12, 20). This process has resulted in a gradient of solutes in the water column that is further stabilized by a perennial ice cover that is 3 to 5 m thick (25, 28). The mixolimnion of the water column is supersaturated with oxygen, while the monimolimnion is anoxic due to limited gas exchange imposed by the perennial ice cover. The latter also serves as a barrier to light penetration, allowing only a small amount of light to reach the water column; for example, in Lake Fryxell, photosynthetically active radiation (PAR) is reduced by more than 90% from a depth of 4 to 9 m and by another 90% from 9 to 11 m (2, 23).

There have been extensive studies of the geochemistry of Lake Fryxell, but to date only a few studies of the microbial communities that inhabit the lake have been reported (16, 24). These studies focused on ammonia-oxidizing bacteria (members of the γ and β subdivisions of the *Proteobacteria*) within the water column and the contributions of cyanobacteria inhabiting the lake's ice cover to primary production in the dry valley ecosystem (6, 29). Studies of the bacterial diversity of microbial mat samples collected from moats that surround the lake during the austral summer season showed a large degree of microbial diversity, including both gram-positive and gram-negative bacteria, as well as *Archaea* (4).

The presence of a year-round ice layer on Lake Fryxell prevents active mixing and maintains an extensive anoxic zone containing sulfide, which is suitable for development of anoxy-

genic phototrophic purple bacteria (21, 23). These organisms, although phototrophic, do not evolve oxygen and are members of the α , β , and γ subdivisions of the *Proteobacteria* (10, 11). The metabolic diversity of purple bacteria, particularly purple nonsulfur bacteria, allows them to occupy a broad range of environments (10, 11). Despite low light intensities in the water column under the ice, photosynthesis occurs in Lake Fryxell, and the contribution of anoxygenic photosynthesis to autotrophic processes in the lake has previously been noted (21).

The *pufM* gene encodes a pigment-binding protein in the photosynthetic reaction center of all purple phototrophic bacteria, as well as *Chloroflexus* (1). This gene is part of the *puf* operon, whose primary transcripts undergo differential decay (9). In classical anoxygenic phototrophs, the *puf* operon is repressed by oxygen and only weakly repressed by light (8), whereas in aerobic anoxygenic phototrophs, expression of the *puf* operon is not sensitive to oxygen but is strongly repressed by high light intensities (15, 19). Previous work has shown that phototrophic purple bacteria but not green bacteria inhabit Lake Fryxell (1). To assess the biodiversity, metabolic activity, and ecology of these phototrophs, a PCR primer set targeting a 228-bp region at the 3' end of the *pufM* gene was employed to screen total genomic DNA and RNA isolated throughout the water column (1). We report here that based on *pufM* and microbiological analyses, a broad diversity of purple nonsulfur bacteria inhabit Lake Fryxell.

MATERIALS AND METHODS

Sampling and water analyses. Water samples were obtained from various depths of the water column of Lake Fryxell in November 1999 and November 2001 through sampling holes that were drilled to within 0.5 m of the ice-water interface with a 4-inch steel drill bit attached to a gasoline-powered mechanical Jiffy drill. The 4-inch hole was further melted out to a diameter of 0.7 m over the course of 2 days by using a Hotsy ethylene glycol circulating heater. The ice depths at the two sampling holes were 4.5 m (1999) and 5.75 m (2001). The finished sampling holes were covered with either a heated Polarhaven weather shelter or an unheated Scott tent, and samples were collected at intervals over a 3-week period. The global positioning system coordinates of one of the holes

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were 77°36.570'S, 163° 08.969'E; the second hole was 100 m northeast of the first hole. Water samples were collected in a 5-liter Niskin bottle and transferred in dim light to completely filled plastic bottles that were stored in the dark and prevented from freezing. Samples were maintained at 5°C until they were processed for nucleic acid extraction or used for enrichment culture. Temperature and dissolved oxygen measurements were obtained using a Yellow Springs Instrument Co. model 57 precalibrated probe. Sulfide concentrations were measured colorimetrically using the methylene blue method as described by Trüper and Schlegel (27). Immediately upon retrieval of water samples, the sulfide at each depth was trapped as ZnS by adding 1 ml of lake water to 3 ml of a 2% zinc acetate solution. Sulfide concentrations were determined using a Beckman DU spectrophotometer at 670 nm and a standard curve prepared from washed crystals of Na₂S · 9H₂O precipitated with Zn to form ZnS.

Enrichment and absorption spectra of phototrophic purple bacteria. Enrichment cultures were established in three different media: (i) medium AB, a malate-mineral salts medium for purple nonsulfur bacteria (14); (ii) medium Pf-7, a mineral salts-sulfide medium suitable for growth of phototrophic purple or green bacteria (30); and (iii) a 1:2 mixture of medium AB and medium Pf-7. The final pH of all media was 7.2. Sterile medium in 10 to 17 ml screw-cap tubes was inoculated with 5 ml of Lake Fryxell water from a given depth, and the tubes were completely filled and sealed. The tubes were incubated at 5°C in dim incandescent light (5 to 10 μmol of photons·m⁻²·s⁻¹) for several months.

Absorption spectra were obtained for intact cells suspended in precooled 30% bovine serum albumin with a Hitachi U-2000 double-beam spectrophotometer.

Nucleic acid extraction. To concentrate the biomass, 500 ml of lake water was filtered through sterile 0.2-μm-pore-size Nalgene analytical test filters. The filters were aseptically cut into approximately 2-mm² pieces and transferred to a biopulverizer tube (Qbiogene, Carlsbad, Calif.) for extraction of either total environmental DNA or RNA. For DNA extraction, biopulverizer tubes containing lysing matrix E, 978 μl of sodium phosphate buffer, 122 μl of MT buffer (Fast DNA Spin kit for soil [Qbiogene]), and test filters were processed with a Mini-Bead Beater (Biospec Products, Bartlesville, Okla.) for 30 s at 4,600 rpm. The remainder of the DNA extraction procedure was performed according to the manufacturer's instructions (Qbiogene). For extraction of RNA, biopulverizer tubes containing lysing matrix B (Qbiogene), 1 ml of RNA Wiz (Ambion, Austin, Tex.), and test filters were processed with a Mini-Bead Beater for 30 s at 4,600 rpm. The remainder of the RNA extraction procedure was performed according to the manufacturer's instructions for RNA Wiz. DNA and RNA samples were analyzed by agarose gel electrophoresis to assess their integrity.

PCR and reverse transcription-PCR. A portion of the *pufM* gene was amplified from total environmental DNA by PCR using the *pufM* primer set (primers 557F [5'-CGACCTGGACTGGAC-3'] and 750R [5'-CCCATGGTCCAGCG CCAGAA-3']) as previously described (1). Total environmental RNA was treated with DNase (DNA free; Ambion) prior to reverse transcription-PCR to prevent amplification of contaminating DNA. To test the efficiency of the DNase treatment, RNA samples were used as templates in a standard PCR amplification with the *pufM* primers. The lack of an amplification product implied that contaminating DNA was not present in the RNA preparations. Reverse transcription was performed with an RNA PCR kit (Applied Biosystems, Foster City, Calif.). The components of the reaction mixture (total volume, 20 μl) were as follows: 5 mM MgCl₂, 1× PCR buffer II, 2 μl of diethyl pyrocarbonate-treated water, each deoxynucleoside triphosphate at a concentration of 1 mM, 1 U of RNase inhibitor, 2.5 U of murine leukemia virus reverse transcriptase, 1 μl of reverse primer (concentration, 125 μg/ml), and 1 μl (≤1 μg) of total RNA. Reverse transcription was carried out at the following parameters: 15 min at 42°C, 5 min at 99°C, and 5 min at 5°C. For subsequent PCR amplification of cDNA, the following reaction components were added to the reverse transcription-PCR tube: 4 μl of 25 mM MgCl₂, 8 μl of 10× PCR buffer II, 1 μl of forward primer (concentration, 125 μg/ml), 2.5 U of AmpliTaq DNA polymerase per 100 μl, and enough water to bring the volume to 100 μl. The cycling parameters were as follows: initial denaturation for 105 s at 95°C, followed by 35 cycles of 15 s at 95°C and 30 s at 60°C and then a final extension at 72°C for 7 min. The resulting amplified cDNA products were analyzed on a 2% agarose gel to ensure a correct size (228 bp) and eluted from the agarose with a GeneClean Spin kit (Qbiogene) for use in denaturing gradient gel electrophoresis (DGGE).

DGGE and sequencing. Four gel-purified *pufM* PCR mixtures were combined (total volume, 200 μl) and concentrated to 10 μl with a DNA SpeedVac for loading on a DGGE gel. Samples were electrophoresed on a 10% acrylamide-bisacrylamide gel prepared from 40% acrylamide-bisacrylamide (38.93 g of acrylamide and 1.07 g of bisacrylamide dissolved in 100 ml of double-distilled water) with 20 to 80% denaturant (100% denaturant was 7 M urea and 40% [vol/vol] formamide) at 130 V for 8 h, using a DCode universal mutation detection system (Bio-Rad, Hercules, Calif.). The DGGE gels were stained with SYBER green I

(Sigma, St. Louis, Mo.) and were visualized on a UV transilluminator. The resulting bands were excised from the gel and eluted in tissue culture water at 4°C overnight. The eluted DNA was then reamplified using the *pufM* primer set and cloned into the pCR 2.1-TOPO vector (Invitrogen, Carlsbad, Calif.). Sequences of the *pufM* inserts were generated using vector primers (M13F and M13R) and a ThermoSequenase cycle sequencing kit (U.S. Biochemicals, Cleveland, Ohio).

Sequence analyses. Using SeqApp (7), Lake Fryxell *pufM* clone sequences were manually aligned with GenBank *pufM* sequences from known purple bacteria. The nucleotide alignment was used to create phylogenetic trees with the PAUP* software package (beta version 4.0) (26). To prevent biases imposed by short sequences, a neighbor-joining tree was generated with the Kimura two-parameter method, using 776 nucleotide positions of the *pufM* gene from cultured anoxygenic phototrophs. Using the tree described above as a topological constraint, a neighbor-joining tree was then generated by inserting the sequences obtained in this study for 228 nucleotide positions of the *pufM* gene. This method of tree generation provided a topology which was more consistent with the topology of trees generated using 16S rRNA sequences than with the topology of trees generated using only truncated sequences. Similarity to *pufM* sequences from known anoxygenic phototrophs was determined using the BlastN function of BLAST (3) and the show distance matrix option in PAUP*.

Nucleotide sequence accession numbers. Sequences generated during this study have been deposited in the GenBank database under accession numbers AY177968 to AY178006. *pufM* sequences from the following cultured organisms were used in the phylogenetic analysis: *Rhodobacter blasticus* (accession number D50649), *Rhodobacter sphaeroides* (X63405), *Rhodobacter capsulatus* (Z11165), *Rhodovulum sulfidophilum* (AB020784), *Rubrivivax gelatinosus* (U51298), *Roseateles depolymerans* (AB028938), *Acidiphilium acidophilum* (AB013379), *Rhodoferrax fermentans* (D50650), *Rhodoferrax antarcticus* (AF333236), *Rhodomicrobium vannielii* (AB101830), *Rhodocyclus tenuis* (D50651), *Chromatium vinosum* (D50647), *Thiocystis gelatinosa* (D50653), *Rhodospirillum molischianum* (D50654), *Ectothiorhodospira shaposhnikovii* (AF018955), *Bradyrhizobium* sp. strain ORS278 (AF182374), *Sphingomonas ursincola* (AB031016), and *Chloroflexus aurantiacus* (X07847).

RESULTS AND DISCUSSION

Amplification and DGGE analysis of the *pufM* gene from the Lake Fryxell water column revealed several phylotypes at all depths sampled (Fig. 1). Sequence analysis of these phylotypes yielded 33 unique *pufM* clones that clustered into six distinct groups representing both the α and β subdivisions of the *Proteobacteria* (Fig. 2). Several of the clone sequences differed by only a few bases, and it was unclear if the differences were a result of polymerase errors during PCR amplification and sequencing. However, many of the changes occurred in the third position, and several identical sequences were detected in more than one DGGE lane, suggesting that the sequence differences were indeed real and not PCR artifacts. Four clone groups (groups A, B, C, and E) showed sequence similarities to cultured purple nonsulfur bacteria (α and β subdivisions of the *Proteobacteria*), whereas clone groups D and F showed sequence similarities to *pufM* genes from various species of aerobic phototrophs (α subdivision of the *Proteobacteria*). Surprisingly, no *pufM* sequences obtained from Lake Fryxell showed similarity to the *pufM* genes of purple sulfur bacteria. Moreover, only one clone group (group E) contained a *pufM* sequence exhibiting more than 90% sequence similarity to the *pufM* gene of a cultured purple nonsulfur bacterium, *Rhodoferrax antarcticus* (14), suggesting that novel purple bacteria inhabit Lake Fryxell.

Many of the clone groups were localized at specific depths in Lake Fryxell, likely a result of the steep physiochemical gradients of light and oxygen that exist there (Fig. 3). Group A contained the largest number of clones and was present throughout the water column; the broadest diversity of group

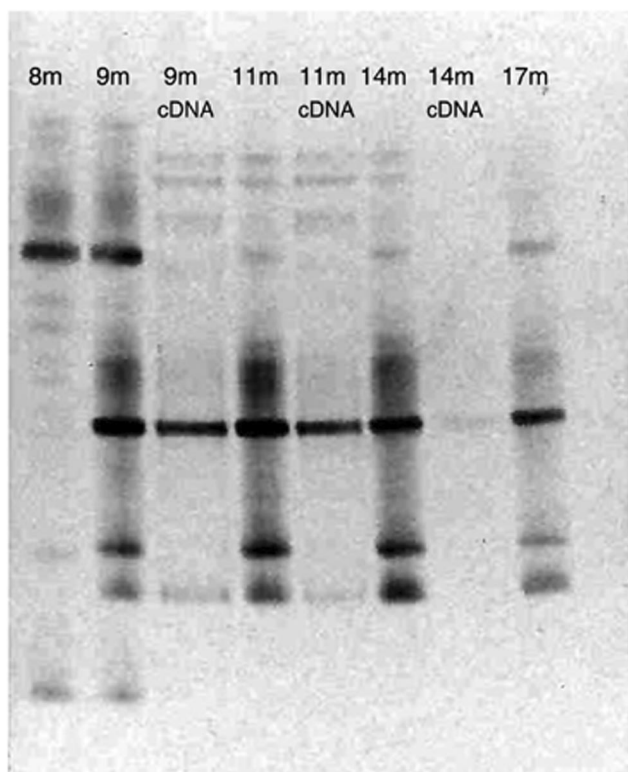


FIG. 1. DGGE gel of Lake Fryxell *pufM* amplification products. Sample designations are indicated at the top.

A clones was located at 9 and 11 m (Fig. 3). Group E clones were also found throughout the water column. Group B phylotypes were found in both shallow (8 and 9 m) and deep (17 m) water, but interestingly, they were not detected in middle depths (Fig. 3). As might be expected, the *pufM* phylotypes showing the highest level of sequence similarity to aerobic anoxygenic phototrophs (groups D and F) were confined to oxic zones of the lake (Fig. 3).

In addition to light and oxygen gradients, there was also a steep gradient of sulfide in Lake Fryxell (Fig. 3). Since purple nonsulfur bacteria, despite their name, are capable of using sulfide as an electron donor and sulfide tolerances are species specific (10), sulfide concentrations could also play a role in the observed distribution of *pufM* phylotypes. For example, although group C *pufM* phylotypes did not exhibit sequence similarity to aerobic phototrophs, these phylotypes were detected only at depths above 9 m, where sulfide was absent, suggesting that these organisms may be species of purple nonsulfur bacteria with a low sulfide tolerance. In contrast, the organisms representing the *pufM* phylotypes found in the 17-m samples may be adapted to high levels (>1 mM) of sulfide and extremely low light intensities (Fig. 3).

The apparent absence of *pufM* signatures of purple sulfur bacteria is an enigma considering the abundance of sulfide in Lake Fryxell (Fig. 3). It is possible that because *pufM* has likely undergone lateral transfer (18, 22) and is thus not as reliable a phylogenetic marker as 16S rRNA, some of the *pufM* phylotypes in Lake Fryxell, especially those at greater depths where sulfide is most abundant, are indeed purple sulfur bacteria.

However, neither molecular (1) nor enrichment evidence for the presence of purple or green sulfur bacteria has been obtained from Lake Fryxell, despite what should be ideal conditions for their development. Long-term (>2-year) incubation of sulfide-containing enrichment cultures failed to reveal such organisms, and thus, if purple and green sulfur bacteria inhabit Lake Fryxell, they are not readily culturable species. In this regard, Lake Fryxell stands in contrast to lakes in the Vestfold Hills area of eastern Antarctica, where both purple and green sulfur bacteria, as well as purple nonsulfur bacteria, are present (5).

Functional expression of *pufM* mRNA transcripts, using reverse transcription-PCR to produce cDNA, was performed to determine which phylotypes were actively transcribing *pufM* in Lake Fryxell. Interestingly, despite the fact that the PufM protein can be produced during chemotrophic growth in the dark (8), *pufM* cDNA was detected only in the 9-, 11-, and 14-m samples (Fig. 1). The greatest diversity of phylotypes producing *pufM* transcripts were present at depths between 9 and 11 m, where light intensity was highest within the nearly anoxic zone (Fig. 3). Sequence analysis of the cDNA showed that group A was the only group expressing *pufM* mRNA and thus likely the only group that was photosynthetically active at the time of sample collection (Fig. 3). Since lake water samples

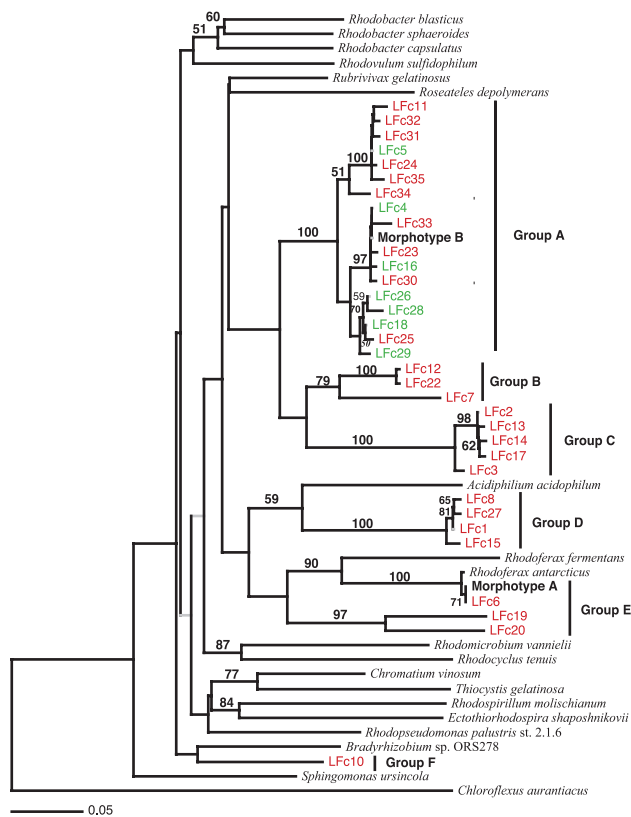


FIG. 2. Neighbor-joining tree based on 228 nucleotide positions of the *pufM* gene, obtained using *Chloroflexus aurantiacus* as the outgroup (see Materials and Methods). All sequences beginning with LF are from Lake Fryxell. Phylotypes in red type represent *pufM* genomic DNA, while phylotypes in green type represent cDNA sequences. Bootstrap values that are >50% are indicated at the nodes. Scale bar indicates the number of substitutions per site.

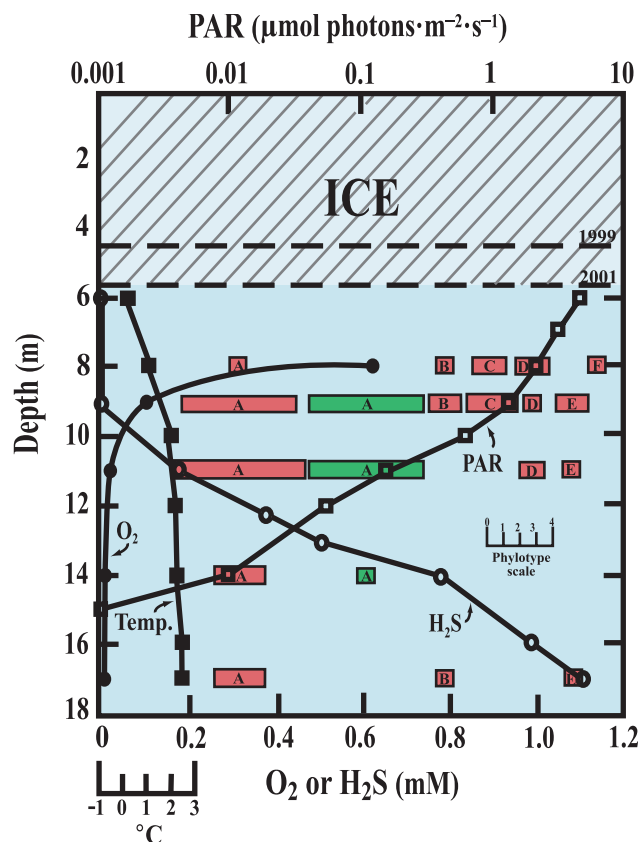


FIG. 3. Profile of Lake Fryxell, summarizing key physiochemical parameters and the locations of different groups of *pufM* phylotypes in the water column. Red, *pufM* genomic DNA; green, *pufM* cDNA. The widths of the boxes are proportional to the number of different phylotypes residing at different depths according to the scale bar. Figure 2 shows the phylotypes in each group.

were taken early in the austral summer, it is possible that other populations of purple bacteria are photosynthetically active only in mid to late summer. Alternatively, phylotypes that did not yield *pufM* mRNA may rely on nonphototrophic forms of energy metabolism. Indeed, perhaps a major reason why purple nonsulfur bacteria rather than purple sulfur bacteria appear to dominate in Lake Fryxell is that typical purple nonsulfur bacteria are metabolically diverse; various species can grow in the dark by chemoorganotrophic or chemolithotrophic metabolism (13). Thus, the physiology of phylotypes residing at great depths, where PAR levels approach zero and *pufM* transcription is undetectable (Fig. 3), may be periodically or permanently nonphototrophic. This capacity for diverse dark metabolism may provide purple nonsulfur bacteria with a competitive edge over purple sulfur bacteria when light is limiting for photosynthesis and may support slow growth in the dark during the extended darkness of the Antarctic winter.

To complement the *pufM* analyses, enrichment cultures for anoxygenic phototrophic bacteria were established. Microscopic observations of positive enrichments revealed two dominant morphotypes of purple nonsulfur bacteria. The first morphotype, designated morphotype A, was a long rod-shaped to filamentous bacterium that contained irregularly shaped, highly refractile regions within cells, reminiscent of clusters of gas vesicles (Fig. 4). The second morphotype, morphotype B, was

distinct from the first morphotype and consisted of club-shaped rods that also displayed refractile areas, typically at one or both poles of the cell (Fig. 4). Transmission electron micrographs of thin sections of morphotype B cells confirmed the presence of gas vesicles (Fig. 4). Although over 15 genera of purple nonsulfur bacteria scattered among both the α and β subdivisions of the *Proteobacteria* are known (10), until now no gas vesiculate species of purple nonsulfur bacteria have been cultured or described. Unless the unusual environment (low temperature, low light) of Lake Fryxell has in some way selected for the gas vesiculate phenotype, the presence of buoyant purple nonsulfur bacteria in other aquatic habitats should therefore be expected.

Absorption spectra of both Lake Fryxell phototrophs clearly showed the presence of bacteriochlorophyll *a* and carotenoids, thus confirming that the organisms were purple bacteria. Maxima at 798 and 836 nm for intact cells of the buoyant morphotype (Fig. 4) indicated that this organism synthesizes

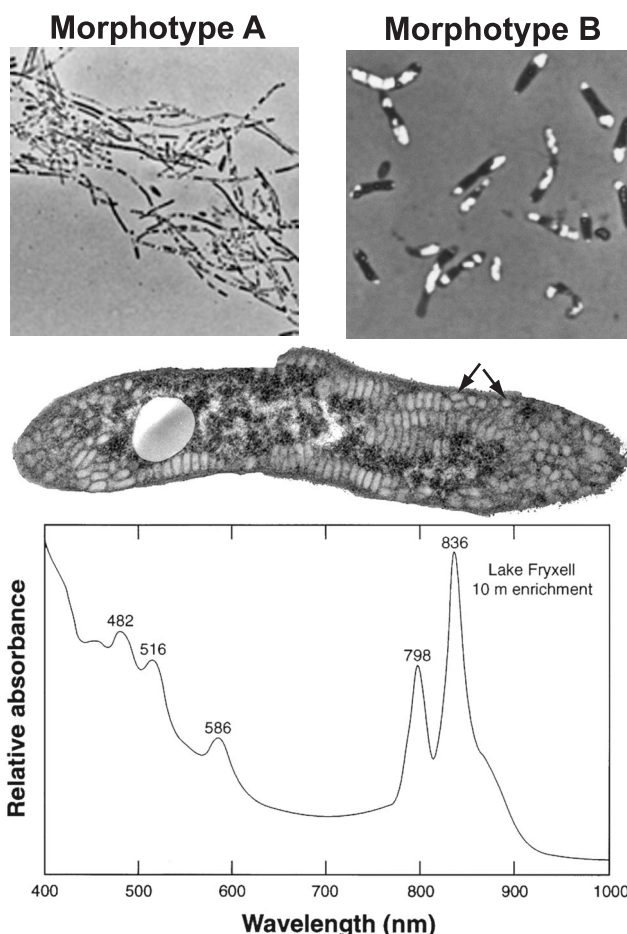


FIG. 4. Enrichment cultures of purple bacteria from a depth of 10 m in Lake Fryxell. The rods and short filaments are morphotype A (a member of group E in Fig. 2). The club-shaped rods are morphotype B (a member of group A in Fig. 2). Also shown (middle panel) is a transmission electron micrograph of a morphotype B cell. Note the abundant gas vesicles (arrows). The spectrum (bottom panel) is the absorption spectrum of intact morphotype B cells; such cells dominated the population in enrichment cultures established from water collected from Lake Fryxell at a depth of 10 m.

a light-harvesting II (peripheral antenna) photocomplex. The shoulder in the spectrum at ~875 nm was likely due to absorbance of a light-harvesting I (core antenna) complex. Methanol extraction of cells gave a sharp peak at 770 nm, confirming that the bacteriochlorophyll was indeed bacteriochlorophyll *a*. Amplification and DGGE analysis of *pufM* genes from the enrichment cultures yielded two *pufM* phylotypes (Fig. 2). Interestingly, the *pufM* genes of morphotypes A and B were also detected after direct *pufM* amplification from Lake Fryxell genomic DNA (groups E and A, respectively) (Fig. 2). Morphotype B is particularly interesting since *pufM* cDNA analysis indicated that it is potentially photosynthetically active in situ and is a member of a clone group for which there is no *pufM* sequence similarity to cultured purple bacteria.

From the molecular and microbiological data presented here, it is clear that a diverse and novel community of purple bacteria inhabits Lake Fryxell. The great diversity of these organisms may be what allows them to successfully inhabit all depths of the water column in this permanently frozen Antarctic lake. The presence of gas vesicles suggests that at least some of these purple nonsulfur bacteria have acquired a means of positioning themselves at specific locations in the near-freezing water column, presumably at depths where the conditions for their growth are optimal. Because purple bacteria are efficient autotrophs (10), this large group of phototrophic bacteria likely contributes organic matter to the anoxic zones of Lake Fryxell. Further investigation and isolation of pure cultures of these purple bacteria should yield important information regarding photosynthesis in the cold and the specific role that phototrophic metabolism plays in the ecosystem of Lake Fryxell.

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