Diversity of Anoxygenic Phototrophs in Contrasting Extreme Environments

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ABSTRACT

This chapter describes the general properties of several anoxygenic phototrophic bacteria isolated from extreme environments. These include purple and green sulfur bacteria from Yellowstone and New Zealand hot springs, as well as purple nonsulfur bacteria from a permanently frozen Antarctic lake. The collective properties of these extremophilic bacteria have yielded new insights into the adaptations necessary to carry out photosynthesis in constantly hot or cold environments.

Key Words

Antarctic Dry Valleys

Chlorobaculum (Chlorobium) tepidum

purple bacteria

Rhodoferax antarcticus

Roseiflexus sp.

Thermochromatium tepidum

1.0 INTRODUCTION

Anoxygenic phototrophic bacteria inhabit a variety of extreme environments, including thermal, polar, hypersaline, acidic, and alkaline aquatic and terrestrial habitats (Madigan 2003). Typically, one finds that these "extremophilic" phototrophs are optimally adapted, or nearly so, to the *in situ* conditions of their habitats (Madigan 2000; Madigan and Marrs 1997; Madigan and Oren 1999). For this and other reasons, certain species of extremophilic phototrophic bacteria have emerged as model systems for defining the physiochemical limits to photosynthetic life. Moreover, pure cultures of these organisms represent valuable genetic resources for future solutions to applied problems in photosynthesis—including, in particular, global warming (see Section 3.1.2).

In this chapter we will discuss several extremophilic phototrophs, with a focus on representatives isolated in this lab from hot spring and polar habitats. These seemingly disparate habitats actually have much in common, including relatively constant (albeit extreme) conditions; a steady supply of resources; restricted microbial diversity; and the absence of "higher" organisms. The chapter opens with discussion of the thermophilic (temperature optimum about 50°C) purple and green bacteria *Thermochromatium tepidum* and *Chlorobaculum* (previously *Chlorobium*) *tepidum*, respectively. It then moves to unpublished data on a newly isolated filamentous and thermophilic phototroph related to *Roseiflexus*. The chapter concludes with a consideration of purple bacteria in permanently frozen Antarctic lakes—the "contrast" to the thermophilic phototrophs whose discussion precedes them.

2.0 MATERIALS AND METHODS

2.1 Organisms

T. tepidum (Madigan 1984, 1986, 2001, 2003) and *Roseiflexus* sp. (M van der Meer, D. Ward, and M. Madigan, unpublished) were isolated from Yellowstone hot springs in the Mammoth Upper Terraces and Octopus Spring (Lower Geyser Basin), respectively. *C. tepidum* (Wahlund et al. 1991) was originally isolated from New Zealand hot springs, but also resides in certain Yellowstone hot springs. Enrichment and isolation procedures for each organism can be found in the references cited.

Antarctic purple bacteria were enriched using standard liquid enrichment methods (Madigan 1988) from the water column of Lake Fryxell, a permanently frozen lake in the Taylor Valley, McMurdo Dry Valleys, Antarctica (Karr et al. 2003).

2.2 Culture Media

Media for cultivation of *T. tepidum* have been described in Madigan (1986), for *C. tepidum* in Wahlund et al. (1991), and for Antarctic purple bacteria in Karr et al. (2003) and Jung et al (2004). *Roseiflexus* sp. was grown in the *Chloroflexus* medium described in Madigan et al. (1974), containing 0-0.3 mM sulfide and 0.1% yeast extract, pH 7.

2.3 Other Methods

Light and electron microscopy and absorption spectra were performed as previously described (Madigan et al. 2000). Lipid analyses were as described in van der Meer et al. (2001). All methods used in the Antarctic study, including molecular, cultural, and field methods, are described in Karr et al. (2003) and Jung et al. (2004).

3.0 RESULTS

3.1 *Thermochromatium tepidum*: The First Thermophilic Purple Bacterium

Purple sulfur bacteria (family Chromatiaceae) are common inhabitants of sulfide-containing springs exposed to light (Madigan 1988); thus, their presence in thermal sulfide springs was not unexpected. The first indication that purple bacteria inhabited thermal springs came from the Japanese botanist Miyoshi. He showed that several Japanese warm springs (40–60°C) supported blooms of purple bacteria, and that sulfide was associated with their development (Miyoshi 1897). Decades after Miyoshi's work, purple sulfur bacteria resembling species of *Chromatium* were identified in several Yellowstone thermal springs (van Niel and Thayer 1930; Castenholz 1969, 1977). These springs are neutral to slightly acidic and are sulfidic, containing 30-300 μM sulfide. Purple sulfur bacteria are distinctive and easy to recognize in natural samples because their pigmented cells contain intracellular globules of elemental sulfur produced from the oxidation of sulfide (**Figure 1A, next page**). Van Niel and Thayer (1930) identified these

organisms only as "purple bacteria" and observed them in both Mammoth and West Thumb hot springs. Interestingly, their report stated that purple bacteria could be found up to 76°C (Van Niel and Thayer 1930). By contrast, later studies by Castenholz (1969, 1977) and Madigan (1984, 1986) have documented these organisms only up to 57°C.

In the early 1980s the Madigan lab set out to culture potentially thermophilic purple bacteria after sulfide/carbonate springs were described in the Mammoth Upper Terraces that contained dense populations of purple sulfur bacteria (Castenholz 1977). In one small sulfide spring (no longer flowing), coined "Roland's Well" by Dave Ward's research group, a thin but distinctly red-colored mat was present containing primarily *Chromatium*-like organisms (**Figure 1**). The type strain (strain MC) of *Chromatium tepidum* was isolated from Roland's Well (Madigan 1984), and was later formally described as a new species of the genus *Chromatium* (Madigan 1986). However, phylogenetic analyses elevated *C. tepidum* to its own genus, *Thermochromatium* (Imhoff et al. 1998; Imhoff and Madigan 2005; Madigan 2001, 2003).

***3.1.1 Physiology and ecology of* Thermochromatium tepidum.** *T. tepidum* is a mild thermophile (optimum ~50°C) that grows within a temperature range of 37-57°C (Madigan 1986). Physiologically, *T. tepidum* resembles the nutritionally restricted large cell species of the genus *Chromatium* (Trüper 1981). Sulfide is required for growth and the only carbon sources photo-assimilated are acetate and pyruvate (Madigan 1986). Moreover, *T. tepidum* is incapable of dark microoxic growth, in contrast to most other purple sulfur bacteria (Kämpf and Pfennig 1980). However, this is not surprising considering that the *T. tepidum* mat is constantly bathed with fresh sulfide-containing spring water and lacks cyanobacteria (Madigan 1984, 1986). Such a constantly anoxic habitat would not offer opportunities for respiratory growth anyway.

The carbonate springs that support populations of *T. tepidum* are ephemeral due to calcium carbonate deposits that eventually plug up the sources. However, a few thermal springs with properties similar to those of Roland's Well are still flowing in the Mammoth Upper

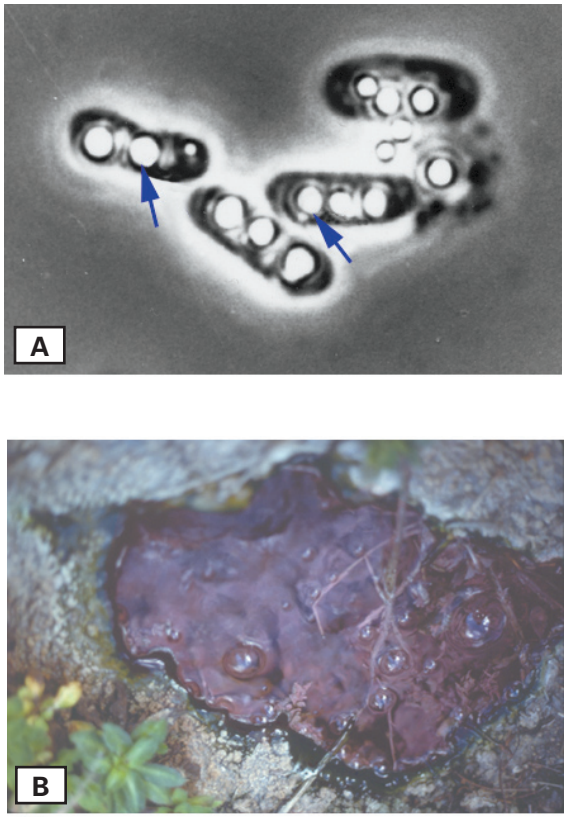


Figure 1. ***Thermochromatium tepidum*. A.** Phase-contrast photomicrograph of cells of *T. tepidum* strain MC. Note sulfur globules (arrows) inside the cells. **B.** "Roland's Well" (unofficial name), a small (0.3 m diameter) sulfide spring in the Upper Terraces of Mammoth Hot Springs, Yellowstone. Spring temperature 55°C, pH 6.5. The bubbles are degassing CO_2 (Photo taken in September 1986). This spring was flowing until the mid-1990s but has since dried up and become overgrown with grass and wildflowers. The red mat contains primarily cells of *T. tepidum*. The type strain of *T. tepidum* (strain MC) originated from Roland's Well.

Terraces and support populations of *T. tepidum* in thin microbial mats. *T. tepidum* coexists in these mats with a *Chloroflexus* species that may be obligately phototrophic (Giovannoni et al. 1987). Collectively, these phototrophs are likely the major primary producers in these habitats, as cyanobacteria are absent (Ward et al. 1989).

A stable-isotope/biochemical study of *T. tepidum* carbon metabolism in these habitats carried out in this laboratory

confirmed this hypothesis: *T. tepidum* employs the Calvin Cycle for CO_2 fixation (Heda and Madigan 1988, 1989), and natural populations of *T. tepidum* cells clearly showed the isotopic signature of autotrophically growing cells (Madigan et al. 1989). Interestingly, however, the *Chloroflexus* populations in these mats showed a stable isotope signature that did not suggest autotrophy, but instead suggested they were incorporating carbon excreted by *T. tepidum*—they were growing photoheterotrophically (Madigan et al. 1989). If true, then *T. tepidum* is the foundation of primary production in these springs and is likely supporting all consumers present in this habitat, including sulfate-reducing bacteria and various other chemoorganotrophic bacteria.

***3.1.2 Photosynthetic properties of* Thermochromatium tepidum.** Following the initial description of *T. tepidum* (Madigan 1986), the focus of attention on this organism quickly turned to its photosynthetic properties, undoubtedly because of its unusual absorption spectrum. *T. tepidum* contains a novel light harvesting (LH) I (core) antenna photocomplex that absorbs maximally near 920 nm (Garcia et al. 1986; Nozawa et al. 1986). This is some 30 nm to the red (that is, to lower energies) of the absorption maximum of the LH I antenna of *Allochromatium vinosum*, a mesophile and a close phylogenetic relative of *T. tepidum* (**Figure 2**). The photosynthetic reaction center of both of these organisms absorbs at 870 nm. Therefore, the back transfer of energy from the core antenna to the reaction center is a significantly greater problem for *T. tepidum* than for *A. vinosum*. Nevertheless, efficient energy transfer from the core antenna to the reaction center in *T. tepidum* has been measured (Kramer and Amesz 1996), although the precise mechanism is unclear.

In addition to studies of the LH photocomplexes of *T. tepidum*, the photosynthetic reaction center of this organism has been crystallized (Nogi et al. 2000). From this work, key amino acid substitutions have been identified that may be responsible for its thermal stability (Nozawa and Madigan 1991). Moreover, the ribulose bisphosphate carboxylase/oxygenase (RuBisCO, Calvin Cycle) of *T. tepidum* has been purified and characterized. *T. tepidum* RuBisCO is stable to 60°C (Heda and Madigan 1988) and

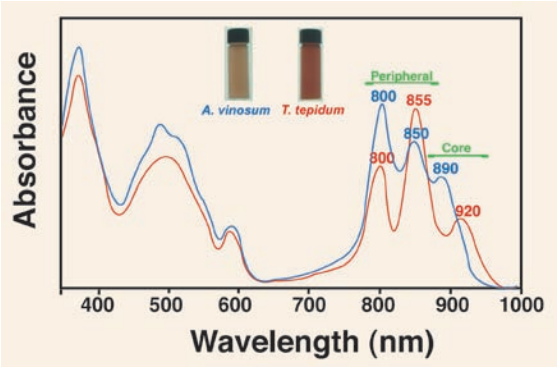


Figure 2. Absorption spectra of intact cells of *Allochromatium vinosum* and *Thermochromatium tepidum*. Cells were suspended in 30% bovine serum albumin. Peripheral (LH II) and core (LH I) antenna absorbance is labeled.

is of the eight large plus eight small subunit type, typical of RuBisCOs from green plants (Heda and Madigan 1989).

The discovery of *T. tepidum* has advanced our understanding of photosynthesis at high temperatures on several fronts—including light energy transfer, thermally stable proteins, and hot spring ecology. To complement this work, the *T. tepidum* genome is currently being sequenced at Integrated Genomics. It is likely that once *T. tepidum's* genetic blueprint is available, it will reveal other molecular secrets for how photosynthesis occurs at high temperature. Of particular interest here will be mechanisms for thermal stability of the peripheral and unusual core antenna complexes of this organism. With global warming looming on the horizon, an understanding of how photocomplexes are made thermally stable might well be useful in an applied sense.

3.2 *Chlorobaculum tepidum*

R.W. Castenholz first discovered microbial mats consisting almost solely of *C. tepidum*, a thermophilic green sulfur bacterium (Wahlund et al. 1991), in the late 1980s in highly sulfidic and acidic hot springs in New Zealand (Castenholz 1988; Castenholz et al. 1990). Recently, similar mats were found in Yellowstone (Bedard et al. 2002). Springs supporting *C. tepidum* mats are apparently rather rare and show an unusual mix of geothermal

conditions. For *C. tepidum* to flourish, there must be sufficiently high levels of sulfide to prevent growth of cyanobacteria and a sufficiently low pH to prevent growth of purple bacteria. In this regard, some *C. tepidum* mats have nearly millimolar levels of sulfide, and pH values as low as 4 (Castenholz 1988; Castenholz et al. 1990).

***3.2.1 Physiology and photosynthetic properties of* Chlorobaculum tepidum.** *C. tepidum* quickly found its way into the mainstream of research on green sulfur bacteria for several reasons including its rapid growth, unique physiological properties, and the availability of genetic exchange systems. Under optimal conditions (pH 6.8, 48°C, incandescent light), cultures of *C. tepidum* grow with a 2-hour doubling time (Wahlund et al. 1991). This is far faster than any species of mesophilic green sulfur bacteria. *C. tepidum* also grows to high cell densities (> 5 x 10^9 cells/mL) in completely autotrophic media. The organism uses thiosulfate as an electron donor, a rarity among green bacteria, and this simplifies growing the organism in large volumes for biochemical work. *C. tepidum* is also an excellent nitrogen-fixing bacterium (Wahlund and Madigan 1993), a rare property among thermophilic prokaryotes. In addition to these ideal physiological properties, genetic transfer systems in *C. tepidum* based on conjugation (Wahlund and Madigan 1995) and transformation (Frigaard and Bryant 2001) are available.

Like all green bacteria, *C. tepidum* contains chlorosomes (**Figure 3**), the light-harvesting structures of green sulfur and green nonsulfur (*Chloroflexus*) bacteria. These giant antenna systems contain a very unusual arrangement of pigments and allow green bacteria to grow at very low light intensities. Unlike the highly differentiated intracytoplasmic membranes of purple bacteria—where photocomplexes are integrated within membranes—bacteriochlorophyll (Bchl) within the chlorosome is not bound to proteins (Olson 1998). The mechanism of energy transfer from chlorosome pigments to reaction center pigments, and chlorosome biochemistry in general, has thus been an active area of research for some time now. But with the discovery of *C. tepidum*, such studies were opened to the powerful tools of molecular genetics. Interestingly,

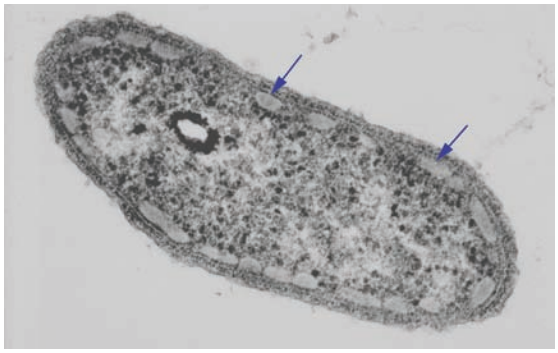


Figure 3. Thin section of a cell of *Chlorobaculum tepidum* strain TLS. Notice the chlorosomes (arrows) lying in the periphery of the cell (Wahlund et al. 1991).

genetic studies have shown that of the 10 proteins that compose the *C. tepidum* chlorosome, mutations in only one generate a lethal phenotype (Frigaard et al. 2004). *C. tepidum* also shows many other unique photosynthetic properties, in particular, the production of novel carotenoid glycosides (Takaichi et al. 1997).

***3.2.2 Phylogeny and genomics of* Chlorobaculum tepidum.** Coupled with its unusual photosynthetic and physiological properties, the unique phylogeny of *C. tepidum* as a member of the Green sulfur bacterial phylum (Wahlund et al. 1991) made it an excellent candidate for genomic analyses. In this connection, the genome sequence of *C. tepidum* was recently completed (Eisen et al. 2002)—the first genome sequence to emerge from any anoxygenic phototroph. Several intriguing findings have come from analyses of the *C. tepidum* genome, including the near total absence of regulatory genes; possession of RuBisCO-like genes that likely play a role in sulfur rather than carbon metabolism; and large numbers of archaeal gene homologs (Eisen et al. 2002). The latter suggests that green sulfur bacteria and species of *Archaea* have shared genes by lateral transfer.

The discovery of *C. tepidum* has opened the era of biochemical genetics in green bacteria. Because green bacteria can grow at the lowest light intensities of all known phototrophs (Kimble and Madigan 2002), the ability to combine genetics with molecular biological analysis of

C. tepidum should allow for even more rapid progress to be made in understanding how photosynthesis occurs at the lower limits of light intensity.

3.3 *Roseiflexus* sp.

Although the filamentous green nonsulfur bacterium *Chloroflexus* has been known for more than 30 years (Pierson and Castenholz 1974), it was not until the age of molecular phylogeny that it became clear that several taxa of these organisms exist. *Chloroflexus*-like organisms reside in hot springs, shallow marine basins, and in freshwater lakes (Pierson and Castenholz 1995). Molecular ecology studies of 16S rDNA of representatives from different habitats have shown that these organisms form a large clade that contains significant phylogenetic breadth (Nübel et al. 2001).

All known *Chloroflexus*-like organisms contain Bchl *c* or *d*, and chlorosomes (Pierson and Castenholz 1995). However, Hanada et al. (2002) described cultures of a filamentous anoxygenic phototroph that contained Bchl *a* but which lacked Bchl *c* and chlorosomes. This organism, *Roseiflexus castenholzii*, was isolated from a Japanese hot spring at Nakabusa (Hanada et al. 2002). Even though *Roseiflexus* retained a phylogenetic link to green nonsulfur bacteria, the organism was sufficiently distinct from *Chloroflexus* to constitute its own genus. Thus, *R. castenholzii* was described as a phototroph, similar in most phenotypic respects to *Chloroflexus aurantiacus*, but lacking the key pigments and chlorosomes of this organism (Hanada et al. 2002).

3.3.1 *A new species of* Roseiflexus *from Yellowstone.*

R. castenholzii-like organisms have also been detected in Octopus Spring by molecular sequencing methods (Boomer et al. 2000, 2002; Nübel et al. 2002). But in addition to *R. castenholzii*, classic *Chloroflexus* signatures have been found as well, along with an organism related to *R. castenholzii*, yet clearly distinct from it. Significantly, this latter organism made up the major proportion of filaments in the microbial mat in the temperature range of 60-70°C (Nübel et al. 2002; Ward et al. 1990; Weller et al. 1992).

Using dilution enrichment techniques we have obtained pure cultures of this *Roseiflexus* relative from Octopus Spring and have referred to the two strains obtained as *Roseiflexus* sp., strains RS1 and RS2. Cultures of either strain are yellow in color, differing from the greenish-brown color of phototrophically grown *Chloroflexus* (**Figure 4A, next page**). The cells are filamentous, resembling *Chloroflexus*, but lack chlorosomes (**Figure 4B**). The *in vivo* absorption spectrum of strain RS1 shows maxima near 800 nm and 880 nm, likely due to a peripheral (LH II) type of Bchl *a* antenna system (**Figure 4C**). The carotenoids of these organisms have not yet been determined.

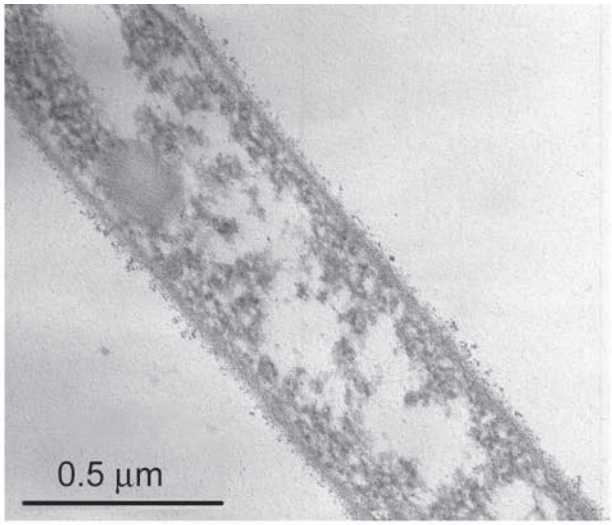
Preliminary phylogenetic analyses of strain RS1 have shown that it is nearly identical in 16S rRNA gene sequence to the "Type C" filamentous phototroph that inhabits the Octopus Spring mat at 60°C (Nübel et al. 2002), and about 95% similar to that of *R. castenholzii* (unpublished results). Physiologically, the organism grows as either a photoheterotroph or an aerobic heterotroph in complex media at an optimum temperature near 55°C. Total lipid extract analysis of RS1 and RS2 showed that the lipid profiles of both isolates are roughly the same. Notably, both organisms contain wax esters similar to those produced by *C. aurantiacus* (van der Meer et al. 2002). Moreover, the wax esters of strains RS1 and RS2 more closely match those of bulk mat material—both in terms of carbon chain length and overall structure—than do the wax esters of either *C. aurantiacus* or *R. castenholzii*. It is therefore possible that strains RS1 and RS2 are more typical of the major components of the Octopus Spring mats than is any organism isolated previously.

Although it is too early to tell what other important properties this new *Roseiflexus* species will show, it is predicted that a detailed study of its physiology will reveal the major physiological loops occurring in the Octopus Spring mat. Of particular interest will be to determine (i) whether strains RS1 and RS2 can grow autotrophically, and if so, which electron donors (H_2, H_2S, $S_2O_3^{2-}$, Fe^{2+}) are used; and (ii) which carbon sources support photoheterotrophic growth.

3.4 Antarctic Anoxygenic Phototrophs

We now consider an environment of great contrast to Yellowstone hot springs: permanently frozen Antarctica lakes. Several freshwater lakes lie in the Taylor Valley,

A



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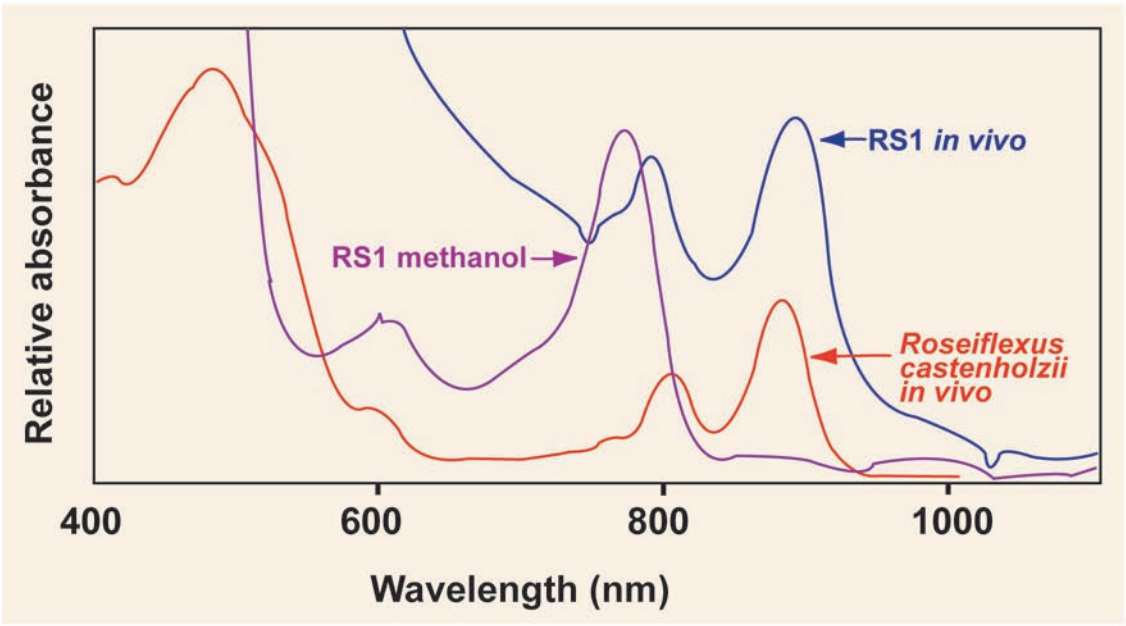

C

Figure 4. ***Roseiflexus*** **species from Octopus Spring, Yellowstone. A.** Mass cultures of *Roseiflexus* sp. strain RS1 (left), and *Chloroflexus aurantiacus* strain J-10 (right). Both cultures were grown phototrophically. **B.** Transmission electron micrograph of a cell of *Roseiflexus* sp. strain RS1. Note the absence of chlorosomes (cells of *Chloroflexus,* by contrast, would contain chlorosomes similar to those shown in the cell in Figure 3). **C.** Absorption spectra of cells of *Roseiflexus* sp. strain RS1 shown with the spectrum of *Roseiflexus castenholzii* for comparison.

McMurdo Dry Valleys, Antarctica. These lakes are highly unusual for several reasons. They are permanently frozen, some with ice covers greater than 6 m, and each has a unique geochemistry (Lyons et al. 1998; Matsumoto 1993). The lakes are also free from wind mixing and are therefore amictic (Howes and Smith 1990). Dry Valley lakes contain a food chain that is exclusively microbial, and experience five months of daylight followed by five months of total darkness each year, with twilight in between. Moreover, some lakes are meromictic from their slightly saline to hypersaline bottom waters (Lyons et al. 1998).

Lake Fryxell (**Figure 5**) is located in the eastern end of the Taylor Valley. The lake supports active sulfur cycling, including sulfate-reduction and sulfur chemolithotrophy (Howes and Smith 1990; Sattley et al. 2003). We have been studying these processes, along with that of anoxygenic photosynthesis that also occurs in this lake. Pigment analyses indicated that purple bacteria are present in Lake Fryxell (Lizotte and Priscu 1998), and we have subsequently confirmed this. The following summarizes our findings.

3.4.1 Molecular profiling of purple bacteria in Lake Fryxell. Molecular methods were used to assess the diversity and physiological activity of purple bacteria in Lake Fryxell. Ribosomal RNA and functional gene analyses showed the lake to be free of detectable green bacteria or heliobacteria, but that purple bacteria were clearly present (Achenbach et al. 2001). Because the phylogeny of purple bacteria is scattered among the Proteobacteria, 16S rRNA methods could not be used to assess diversity in these organisms; instead, a major metabolic gene was chosen. The gene *pufM* encodes a key pigment-binding protein that is only found in the photosynthetic reaction center of purple phototrophic bacteria and *Chloroflexus* (Nagashima et al. 1997). The gene is highly conserved, and, using universally conserved regions, primer sets were constructed that amplified *pufM* from all purple bacteria tested. The PCR product obtained, just 229 bp, could be easily sequenced on a large scale for comparative analyses (Achenbach et al. 2001).

Amplification of *pufM* from Lake Fryxell water samples, followed by denaturing gradient gel electrophoresis

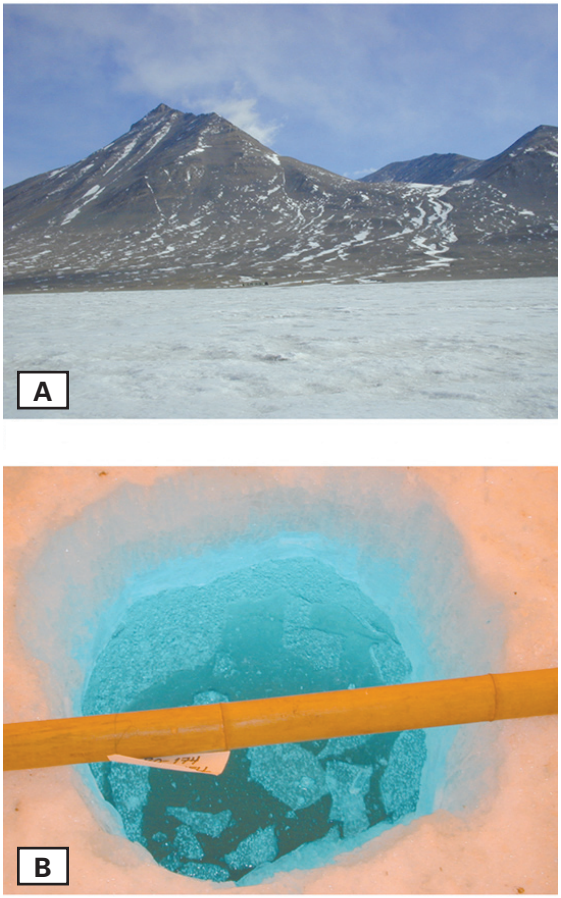

Figure 5. Lake Fryxell, Taylor Valley, McMurdo Dry Valleys, Antarctica. The lake is approximately 2 × 5 km in size. **A.** View looking north from the center of the lake with the Transantarctic Mountains in the background. **B.** Sampling hole drilled through 5.7 m of ice. Once drilled with a 4" mechanical drill, the hole was melted out to about 0.5 m with a circulating system containing hot ethylene glycol.

(DGGE) analysis, revealed several *pufM* phylotypes (sequences). Sequence analysis yielded a total of 33 unique *pufM* clones that clustered into six distinct clades, designated groups A–F, representing both the α and β subdivisions of the Proteobacteria (**Figure 6, next page**). The sequence of a few clones resembled (77-81% similarity) *pufM* sequences from species of aerobic phototrophic bacteria (purple bacteria that carry out photosynthesis only

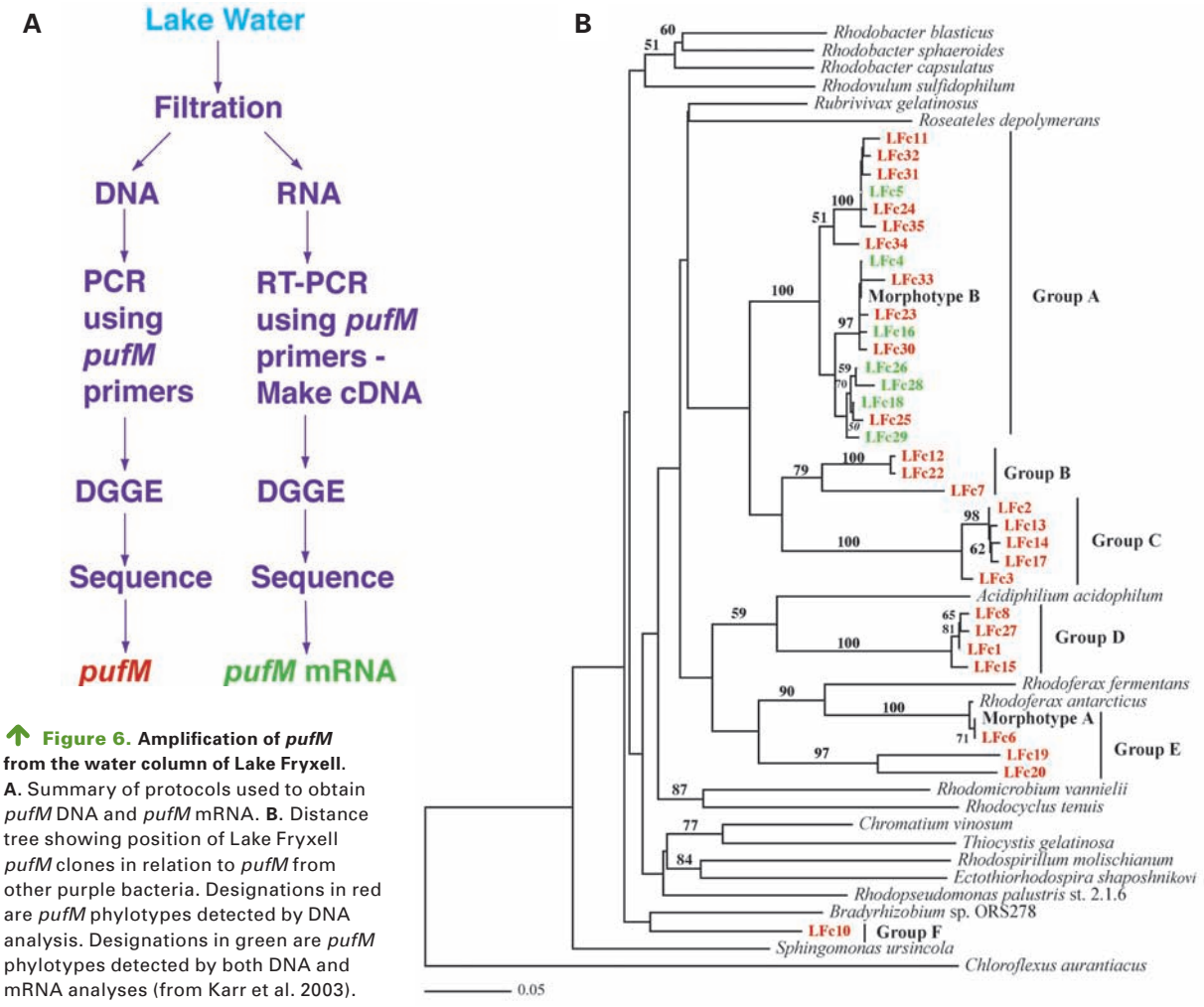

Figure 6. Amplification of *pufM* from the water column of Lake Fryxell. **A.** Summary of protocols used to obtain *pufM* DNA and *pufM* mRNA. **B.** Distance tree showing position of Lake Fryxell *pufM* clones in relation to *pufM* from other purple bacteria. Designations in red are *pufM* phylotypes detected by DNA analysis. Designations in green are *pufM* phylotypes detected by both DNA and mRNA analyses (from Karr et al. 2003).

under oxic conditions; Shimada 1995). But surprisingly, no *pufM* sequences were obtained that showed similarity to those of purple sulfur bacteria (γ-Proteobacteria) despite the presence of significant sulfide in the water column (Karr et al. 2003; Sattley et al. 2003).

In addition to simply identifying the *pufM* phylotypes present in Lake Fryxell, gene expression studies were also performed. Detection of *pufM* message using RT-PCR (reverse transcriptase-PCR, a technique for making cDNA from RNA) identified the phylotypes that were making new photocomplexes. Interestingly, only certain phylotypes could be shown to be active in this regard, and all belonged to Group A (**Figure 6B**), the largest collection of phylotypes discovered. We hypothesize that only this group was carrying out active photosynthesis at the time of sampling (Karr et al. 2003).

3.4.2 Spatial profile of purple bacteria in Lake Fryxell.

Figure 7 shows a depth profile of Lake Fryxell, including several physiochemical parameters. Opposing gradients of sulfide and oxygen are present, typical of meromictic lakes that contain sulfate. Sulfide reached a maximum of 1.1 mM near the sediments. A steep light gradient was also present,

with conditions at depths below about 12 m virtually in darkness.

Figure 7 also summarizes the spatial picture of *pufM* phylotypes with depth, including both the DNA and mRNA data. Group A phylotypes were found throughout the water column, even near the sediments. By contrast, certain phylotypes such as C and F were present only in the upper regions of the water column within the oxic zone and away from the sulfide (**Figure 7**). Group F, a phylotype showing affinity to *pufM* from aerobic phototrophs, is likely positioned such that it can remain photosynthetically active above the sulfidic (anoxic) zone. Expression of *pufM* was limited to cells positioned at 9–11 m; only a single phylotype was producing *pufM* mRNA beneath 11 m (**Figure 7**). It is likely that this organism is an extremely low light-adapted species of purple bacteria.

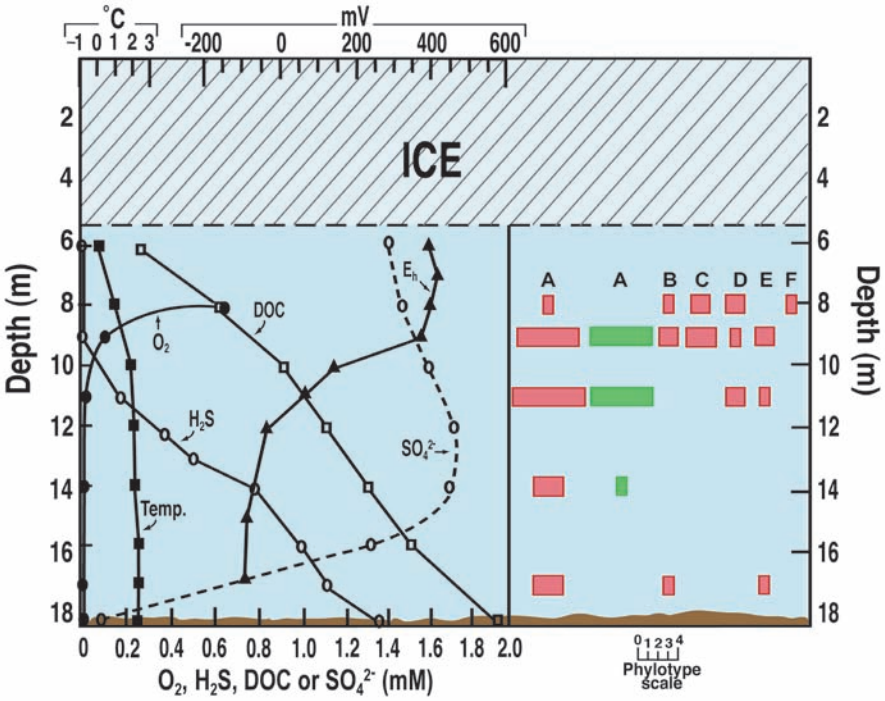

Figure 7. Vertical profile of Lake Fryxell showing light and geochemical parameters and the spatial distribution of pufM phylotypes (depths sampled were 8, 9, 11, 14, and 17 m). The length of each horizontal bar is proportional to the number of different phylotypes present at each depth. Red, *pufM* DNA; Green, *pufM* mRNA. Note how only phylotypes from group A are transcribing *pufM* (adapted from Karr et al. 2003).

3.4.3 Cultures of purple bacteria from Lake Fryxell. In parallel with the molecular experiments (**Figures 6 and 7**), enrichment cultures were established for phototrophic purple bacteria from Lake Fryxell (Karr et al. 2003; Jung et al. 2004). In all enrichments (established at 4°C and low light), one of two different morphotypes of purple nonsulfur bacteria dominated. Morphotype A consisted of long rods to occasional short filaments (**Figure 8A, next page**). Morphotype B was distinctively rod-shaped, resembling a bullet in morphology (**Figure 8C**). Both morphotypes showed irregular refractive areas in the cell, reminiscent of clusters of gas vesicles, and electron micrographs confirmed this. In the bullet-shaped cell, for example, gas vesicles were concentrated at the poles and were also present as a palisade layer along the long axis of the cell (**Figure 8D**).

Absorption spectra of both morphotypes were similar, with major absorption maxima at 836 nm and 798 nm, indicative of Bchl *a*. Methanol extraction of cells gave a sharp peak at 770 nm, confirming that the Bchl present in both morphotypes was indeed Bchl *a* (data not shown). In addition, both morphotypes grew slowly at the enrichment temperature of 4°C. Morphotype A grew more rapidly, and pure cultures were eventually obtained by conventional techniques. Growth experiments have shown that morphotype A grows best at about 18°C and does not grow above 25°C. Furthermore, like its counterpart in morphotype B, the organism contains gas vesicles (**Figure 8B**). Morphotype A has now been

described as a gas vesiculate and planktonic strain of *Rhodoferax antarcticus* (Jung et al. 2004).

4.0 DISCUSSION

4.1 Photosynthesis at High Temperatures

Both *T. tepidum* and the *Roseiflexus* sp. described in this chapter could be considered purple bacteria in that their sole Bchl is Bchl *a*. However, the two species are phylogenetically and physiologically quite distinct. The ecology of *T. tepidum* is clearly linked to sulfide-containing hot springs. Organisms that appear similar to *T. tepidum* can occasionally be seen microscopically in weakly sulfidic alkaline hot spring mats (unpublished observations). But it is only in springs with significant (>30 μM) sulfide that the organism forms blooms. In such springs cyanobacteria are absent, likely because of sulfide inhibition (Castenholz 1977).

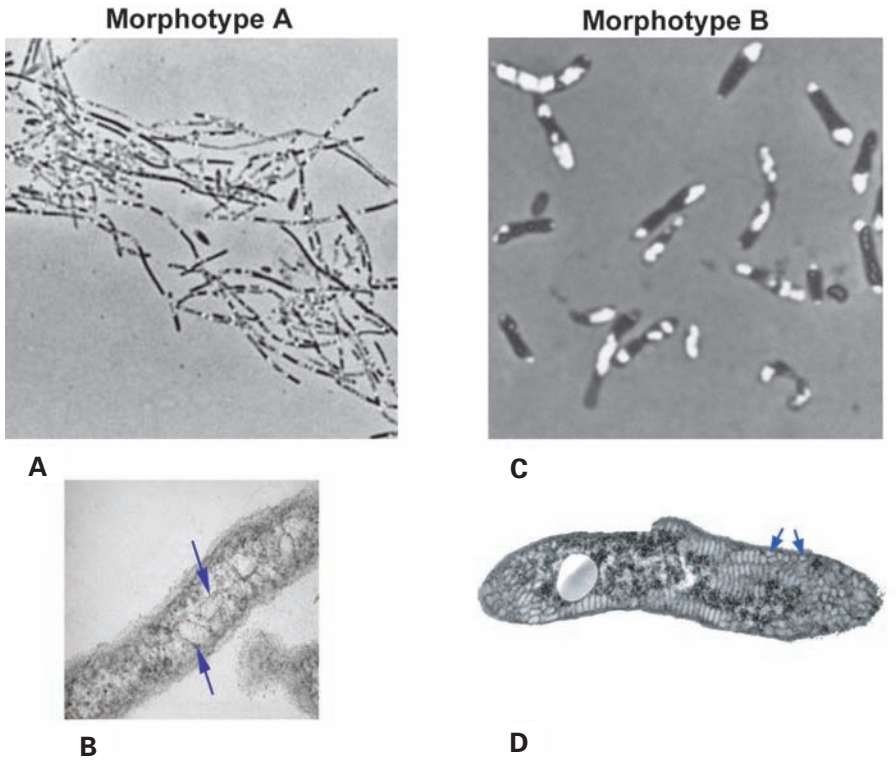

Figure 8. Purple nonsulfur bacteria isolated from Lake Fryxell. A, C, phase-contrast photomicrographs; **B, D,** transmission electron micrographs. Note gas vesicles (phase bright regions in phase-contrast micrographs) in both organisms. Gas vesicles in TEMs indicated by arrows (parts **A, C, and D,** from Karr et al. 2003). Cells of both morphotypes A and B are about 0.5 μm in diameter.

As the pH of sulfidic springs decreases from neutrality to below pH 5.5, and as sulfide levels increase, the springs become habitats for *C. tepidum*. Acidic, moderately thermophilic, high sulfide springs are apparently quite rare, as they were not detected in Yellowstone until the systematic search of Bedard et al. (2002). However, once springs similar to those of the original habitats of *C. tepidum* in New Zealand (Castenholz 1988) were located in Yellowstone, they predictably contained populations of *C. tepidum*. Interestingly, the *C. tepidum* populations in the Yellowstone springs were related to but distinct from those inhabiting New Zealand springs (Bedard et al. 2002).

Studies of the newly isolated *Roseiflexus* species described earlier are in their infancy, and much more work is needed to understand its role in the mat community of Octopus Spring. From the initial experiments here, however, it is clear that both photoheterotrophic and dark heterotrophic growth of this organism is possible. But it is very important to know if this *Roseiflexus* species can grow photoautotrophically (presumably with sulfide or hydrogen as electron donors), as can *Chloroflexus* (Madigan 1988). If photoautotrophy in strains RS1 and RS2 is possible, it will have direct relevance to understanding production and consumption processes in alkaline hot springs such as Octopus Spring. If autotrophy is possible, it will also be interesting to determine the mechanism(s) involved. Related green nonsulfur bacteria such as

C. aurantiacus fix CO_2 by a unique series of reactions, the 3-hydroxypropionate pathway (Herter et al. 2002). In mats where *Roseiflexus* sp. predominates, its lipid biomarkers have heavy ^{13}C signatures, suggesting the possibility that this organism may be capable of photoautotrophy by such a pathway (van der Meer et al. 2000).

4.2 Photosynthesis at Cold Temperatures

The thermal habitats of *T. tepidum*, *Roseiflexus* sp., and *C. tepidum*, stand in stark contrast to the permanently cold habitats of Antarctic lakes. However, our work has shown that Antarctic lakes are suitable habitats for anoxygenic photosynthesis. In the case of Lake Fryxell there appears to be a broad diversity of purple nonsulfur bacteria present, at least as far as differences in *pufM* sequence are a valid measure of diversity.

We hypothesize that up to six "species" of purple nonsulfur bacteria exist in Lake Fryxell, one for each cluster of *pufM* phylotypes (**Figure 6**). Morphotype A has already been characterized as the first gas vesiculate and planktonic purple nonsulfur bacterium (Jung et al. 2004). The *pufM* of morphotype A is almost identical to that of the type strain of *R. antarcticus*, a morphologically-distinct organism isolated from an Antarctic microbial mat. By contrast, the *pufM* sequence of morphotype B clusters with phototrophic species of α-Proteobacteria, suggesting that this organism may belong to this group.

The presence of gas vesicles in morphotypes A and B is noteworthy, as gas vesicles have never been described in species of purple nonsulfur bacteria. Gas vesicles likely have ecological relevance for these phototrophic bacteria. Phototrophic organisms that can maintain their position in the water column nearest the available light and just within the anoxic zone would likely have a selective advantage. And because these lakes lack wind mixing, gas vesicles should be an ideal strategy for maintaining a particular position in the lake for long periods without expending energy for motility. Observations on the distribution of planktonic cyanobacteria in other freshwater lakes are in agreement with this hypothesis (see Jung et al. 2004 for further discussion).

A rather curious finding in this study is the putative absence of purple sulfur bacteria from Lake Fryxell. The physiochemical conditions in this lake, especially the sulfide gradient that exists from zero to more than 1 mM, should provide ideal conditions for growth of purple sulfur bacteria. We have no clear explanation for this, but suggest two possibilities. First, there is a problem with *pufM* as a phylogenetic tool, as it does not track phylogeny as well as does 16S rRNA and has likely undergone lateral transfer (Nagashima et al. 1997). Therefore, it is possible that some of the *pufM* phylotypes identified in the Lake Fryxell water column (**Figure 6**) are actually those of purple sulfur bacteria. If true, these organisms are not readily cultured; cold, sulfide-containing enrichment cultures maintained for over two years have not yielded purple or green sulfur bacteria.

An alternative explanation for the putative absence of purple sulfur bacteria in Lake Fryxell may be the unique limnological conditions found there. In particular, this lake experiences darkness for more than five months each year. Light is a key ecological parameter for phototrophic organisms and may strongly control which groups of phototrophs are most successful in Antarctic lakes. For example, in contrast to purple sulfur bacteria, most of which show only a weak dark metabolism (Kämpf and Pfennig 1980), purple nonsulfur bacteria are typically metabolically diverse, with well-developed dark metabolisms (Madigan 1988). In fact, in some species of purple nonsulfur bacteria, several strategies for dark growth exist, both aerobically and anaerobically (Madigan and Gest 1979; Madigan 1988). It is thus possible that the inherent metabolic versatility of purple nonsulfur bacteria allows them to continue growing in Lake Fryxell during the austral winter. Because of this, over time they may have supplanted purple sulfur bacteria, if the latter have ever been present there.

Arguing against the absence of purple and green sulfur bacteria from Lake Fryxell, however, is the fact that what appear to be purple sulfur bacteria, and what clearly are green sulfur bacteria, exist in the frozen lakes of the Vestfold Hills in East Antarctica (Burke and Burton 1988a, 1988b; Jung et al. 2001). These lakes receive nearly

the same light regime as does Lake Fryxell, but differ in one key respect: Vestfold Hills lakes thaw to open water for one month per year and thus allow for wind mixing (Burke and Burton 1988a, 1988b).

If Lake Fryxell really does contain only purple nonsulfur bacteria, the depth profile shown here (**Figure 7**) indicates that at least some of them must be quite sulfide tolerant. Indeed, preliminary experiments with cultures of morphotype A show this to be the case (growth occurs up to 2 mM sulfide). If it turns out that all of the *pufM* phylotypes detected in Lake Fryxell (**Figure 6**) belong to species of purple nonsulfur bacteria, then some of these species must be remarkably sulfide tolerant. Only a very few purple nonsulfur bacteria can grow at millimolar levels of sulfide (Hansen and van Gemerden 1972). In this regard, studies with the gas vesiculate strain of *R. antarcticus* mentioned previously showed it to be capable of growth in media containing 4 mM sulfide (Jung et al. 2004).

Further work on the biodiversity of anoxygenic phototrophs in Lake Fryxell is clearly necessary if we are to understand the diversity of phototrophs that can thrive there. In this regard, Winogradsky columns have been established using Lake Fryxell sediment and water for long-term cold/low light incubation. If purple/green sulfur bacteria do not emerge from such enrichments—the classical way of obtaining them—it is then likely that Lake Fryxell is truly suitable only for purple nonsulfur bacteria.

4.3 Epilogue: Pure Cultures and Phototrophic Extremophiles

In this era of molecular microbiology, environmental sequences have shown the great diversity of organisms remaining for microbiologists to culture. But pure cultures have a place in modern microbiology, as well. For example, with each organism described in this chapter, something new about photosynthesis has been learned from examining it, something that could not be learned from molecular sequences alone. Cultures of *T. tepidum* revealed the unique core antenna of this organism (Garcia et al. 1986; Nozawa et al. 1986). The discovery of *C. tepidum* reinvigorated research on green bacteria and yielded a genome from the green sulfur bacteria long before it might otherwise have emerged. What exciting science lies in store from cultures of Antarctic purple bacteria? Among other things, it is hoped that these psychrophilic phototrophs will reveal the secrets to constructing cold-active photocomplexes.

Studies of prokaryotic diversity thus need two components. Molecular profiling is needed to assess the extent of the diversity present. But pure culture microbiology is required to determine the major biological properties of the species that are there. Molecular methods and isolation methods thus go hand-in-hand. Either one alone is insufficient for a modern dissection of the prokaryotic diversity of a given habitat. This is a lesson we all need to remember as we search new and diverse habitats for the presence of fascinating new prokaryotes.

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