Microbial Diversity and Bioprospecting
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Chapter 13

Earth's Icy Biosphere

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EARTH'S COLD BIOSPHERE

Earth's biosphere is cold, with 14% being polar and 90% (by volume) cold ocean at <5°C. More than 70% of Earth's freshwater occurs as ice, and a large portion of the soil ecosystem (~20%) exists as permafrost. There is even evidence that bacteria proliferate in high-altitude supercooled cloud droplets (Sattler et al. 2001), using organic acids and alcohols for growth. Microorganisms in sea ice were noted by early sailors and first studied as a scientific curiosity by Bunt (1964). At maximum extent, sea ice covers some 5% of the Northern Hemisphere and 8% of the Southern Hemisphere and accounts for about 67% of the Earth's ice cover. Despite the global significance of sea ice, only recently has it been explored for novel microorganisms (Bowman et al., 1997; Junge et al., 2002; Thomas and Dieckmann, 2002). Recent studies of microbial diversity in polar oceans have shown a predominance of *Archaea* in the subgroup *Chrenarchaeota* (DeLong et al., 1994). Previous to this discovery, *Archaea* were thought to be confined to thermal systems. Paleoclimate records for the past 500,000 years have shown that the surface temperature on Earth has fluctuated drastically, with four major glaciations occurring during this period (Petit et al., 1999). Strong evidence also exists showing that the Earth was completely ice covered during the Paleoproterozoic and Neoproterozoic periods (Kirschvink, 1992; Hoffman et al., 1998). New discoveries of microbial life in cold (−5°C) and saline lakes (Franzman et al., 1997; Priscu et al., 1999a; Takacs et al. 2001), permanent lake ice (Priscu et al., 1998; Fritsen and Priscu, 1998; Psenner et al., 1999), glacial ice (Christner et al., 2000, 2001; Skidmore et al., 2000), and polar snow (Carpenter et al., 2000) are extending the bounds of our biosphere. The recent description of potential bacterial life in Lake Vostok (Priscu et al., 1999b; Karl et al., 1999; Christner et al., 2001) and the discovery of at least 100 other Antarctic subglacial lakes extend the known boundaries for life on Earth even further. Even with the spatial and temporal records for icy systems on Earth, little is known about the psychrophilic or psychrotolerant microorganisms that inhabit them. Despite the mounting evidence for microbial life in frozen ecosystems, many textbooks limit their definitions of the biosphere to the region between the outer portion of the geosphere and the inner portion of the atmosphere, neglecting icy habitats. Clearly, we must extend the bounds of what is currently considered the Earth's biosphere to include icy systems.

COLD EXTRATERRESTRIAL LIFE?

Studies of Earthly ice-bound microbes are also relevant to the evolution and persistence of life on extraterrestrial bodies. This is particularly evident because the average temperature of the Universe is just a few degrees above absolute zero. During the transition from a clement environment to an inhospitable environment on Mars, liquid water may have progressed from a primarily liquid phase to a solid phase, and the Martian surface would have eventually become ice covered (Wharton et al., 1995). Evidence from Martian orbiter laser altimeter images has revealed that water ice exists at the poles and below the surface of Mars (Boynton et al., 2002; Malin and Carr, 1999), and studies of Martian meteorites have inferred that prokaryotes were once present (Thomas-Keprta et al., 2002). Habitats in polar ice may serve as a model for life on Mars (Priscu et al. 1998, 1999a, 1999b; Paerl and Priscu, 1998; Thomas and Dieckmann, 2002) as it cooled and may assist us in our search for extinct or extant life on Mars today. Biochemical traces of life or even viable microorganisms may well be protected from destruction if deposited within polar perennial

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ice or frozen below the planet's surface. During high obliquity, increases in the temperature and atmospheric pressure at the northern pole of Mars (McKay and Stoker, 1989; Malin and Carr, 1999) could result in the discharge of liquid water that might create environments with ecological niches similar to those inhabited by microorganisms in terrestrial polar and glacial regions. Periodic effluxes of hydrothermal heat to the surface could move microorganisms from the Martian subterranean, where conditions may be more favorable for extant life (McKay, 2001). The annual partial melting of the ice caps might then provide conditions compatible with active life or at least provide water in which these microorganisms may be preserved by subsequent freezing (McKay and Stoker, 1989; Clifford et al., 2000). The microfossils and chemical signatures of potential biological origin that were recently discovered in Alan Hills meteorite ALH84001 reinvigorated the debate over the possibility of life on Mars (McKay et al., 1996; Thomas-Keprta et al., 2002). However, such circumstantial evidence will require confirmation by scientific missions to explore and study the frozen surface of Mars.

Surface ice on Europa, one of the moons of Jupiter, appears to exist in contact with subsurface liquid water (Greenburg et al., 2000; Kivelson et al., 2000). Geothermal heating and the tidal forces generated by orbiting Jupiter are thought to maintain a 50- to 100-km-deep liquid ocean on Europa with perhaps twice the volume of the Earth's ocean (Chyba and Phillips, 2001), but beneath an ice shell at least 3 to 4 km thick (Turtle and Pierazzo, 2001). Cold temperatures (<128 K [Orton et al., 1996]) combined with intense levels of radiation would appear to preclude the existence of life on the surface, and the zone of habitability (i.e., where liquid water is stable) may be present only kilometers below the surface where sunlight is unable to penetrate (Chyba and Hand, 2001). Europa's surface appears strikingly similar to terrestrial polar ice floes, suggesting that the outer shell of ice is periodically exchanged with the underlying ocean. The ridges in the crust and the apparent rafting of dislocated pieces imply that subterranean liquid water flows up through stress-induced tidal cracks, which may then offer provisional habitats at shallow depth for photosynthesis or other forms of metabolism (Gaidos and Nimmo, 2000; Greenberg et al., 2000). Gaidos et al. (1999) argue that without a source of oxidants, Europa's subsurface ocean would be destined to reach chemical equilibrium, making biologically dependent redox reactions thermodynamically impossible. However, the surface is continually bombarded with high-energy particles, producing molecular oxygen and peroxides, as well as formaldehyde and other organic carbon sources (Chyba, 2000; Chyba and Hand, 2001); and it is conceivable that Europan microbial life might subsist without employing photosynthetic or chemoautotrophic lifestyles. In this scenario, mixing between the crust and the subsurface need be the only mechanism required to provide organics and oxygen at levels sufficient to support life (Chyba, 2000). Tidal heat generation and electrolysis could also provide sources of energy that could be coupled to bioenergetic redox reactions (Greenberg et al., 2000). The vast network of Antarctic subglacial lakes that lie ~4 km beneath the permanent ice sheet provides an earthly analog in the search for life on Europa and a model system to develop the noncontaminating technologies that will be required to sample Europa.

EVOLUTION OF LIFE ON A FROZEN EARTH

The biology of permanently cold environments on our own planet has received relatively little investigation. Similar to their high-temperature counterparts, frozen ecosystems are dominated by microorganisms. Expectations of commercial applications and interest in the early evolution of life have led many researchers to examine microorganisms, including cyanobacteria, in thermal systems. Based on the deep-rooted phylogeny of thermophilic bacteria and archaea in the tree of life (i.e., small-subunit rRNA phylogeny), in concert with extensive geothermal activity during the early evolution of our planet, it is generally thought that life on Earth evolved in a hot environment (Huber et al., 2000; Pederson, 1997). Recent considerations about the evolution of life, however, have suggested that a "hot start" was probably not the only alternative for the origin of life. Although there are strong arguments for a thermal origin of life based on small-subunit ribosomal RNA (16S/18S rRNA) phylogenetic relationships, the validity of this relationship is questioned by researchers who believe that phylogenies are strongly biased by the use of just a single gene for the construction of the tree of life. If lateral gene transfer is common among all prokaryotic organisms (Nelson et al., 1999), then a hierarchical universal classification is difficult or impossible (Pennisi, 1998, 1999), and evolutionary patterns must be reassessed (Doolittle, 1999). A hot origin of life is also not supported by new results from phylogenetic trees based on genes that do not code for rRNA, chemical experiments with alternative structure for the nucleic acid backbone (Eschenmoser, 1999), considerations about the thermal stability of basic molecules found in all organisms, and statistical analysis of the G+C content of DNA (Galtier et al., 1999). Adaptation to life in hot environments may even have occurred late in evolutionary time (Balter, 1999).

Although much more research is required to determine whether life originated in hot or cold environments, it is highly probable that cold environments have acted as a refuge for life during major glaciations. Recent evidence has indicated that, around 600 million years ago during the Neoproterozoic, early microbes endured an ice age with such intensity that even the tropics froze over (Hoffman et al., 1998; Hoffman and Schrag, 2000; Schrag and Hoffman, 2001). According to this hypothesis, known as the Snowball Earth Hypothesis, the Earth would have been completely ice covered for 10 million years or more, with ice thickness exceeding 1 km. Only the deepest oceans would have contained liquid water. One of the primary criticisms of the Snowball Earth Hypothesis is that the thick ice cover over the world ocean would cut off the supply of sunlight to organisms in the seawater below and thereby eliminate photosynthesis and all life associated with photosynthetic carbon production. Others have concluded that global-scale freezing would extinguish all surface life (Williams et al., 1998). Only the hardiest of microbes would have survived this extreme environmental circumstance, and perhaps icy refuges or hot springs on the seafloor and terrestrial surface may have served as oases for life during these lengthy crises. Hoffman and Schrag (2000), Vincent and Howard-Williams (2001), and Vincent et al. (2002) suggest that photosynthetic cyanobacteria and bacteria, similar to those found in the permanent ice covers of contemporary polar systems, may have acted as an icy refuge during this period, until postsnowball melting introduced conditions suitable for activity in terrestrial and marine habitats. The resultant high concentration of microbes in these icy environments would favor intense chemical and biological interactions between species, which could entice the development of symbiotic associations, and perhaps influence eukaryotic development through evolutionary time (Vincent et al., 2002). Although this "density speeds evolution" theory has been considered primarily in the context of thermal microbial mat communities (Margulis and Sagan, 1997), it is possible that ice-bound habitats also provided opportunities for microbial evolution and the acquired biological innovations may have triggered the Cambrian explosion, which occurred immediately after the last snowball Earth event (Knoll, 1994; Hoffman and Schrag, 2000; Kirschvink et al., 2000). Ironically, the deep-rooted phylogeny of thermophilic species, generally interpreted as evidence for the origin of life under hot circumstances, may instead be the consequence of an evolutionary "bottleneck" imposed by the extremes of multiple snowball Earth events (Kirschvink et al., 2000).

It is clear that a great diversity of icy environments make up Earth's cold biosphere. Given the space constraints of this book, we dedicate the remainder of this chapter to describing research conducted in our own laboratories on the newly discovered life associated with permanent Antarctic lake ice, glaciers and ice sheets (polar and temperate), and subglacial Antarctic lakes.

PERMANENT ANTARCTIC LAKE ICE

The McMurdo Dry Valleys form the largest (~4,000-km^2) ice-free expanse of land on the Antarctic continent. Meteorological conditions in the dry valleys reveal the extreme conditions that organisms must overcome to survive (Priscu et al., in press; Doran et al., 2002a, 2002b, in press). Surface air temperatures average −27.6°C, and there are on average only about 6.2 degree-days per year above freezing (temperature above freezing times the number of days above freezing each year [Doran et al., 2002]). These conditions produce the only permanently ice-covered lakes on Earth. During studies on the biogeochemistry of nitrous oxide in McMurdo Dry Valleys lakes, Priscu and coworkers (Priscu et al., 1996; Priscu, 1997) observed a peak in nitrous oxide associated with a sediment layer 2 m beneath the surface of the 4-m-thick ice cover of Lake Bonney (Priscu et al., 1998). This observation together with elevated levels of chlorophyll *a,* particulate organic carbon, particulate organic nitrogen, ammonium, and dissolved organic carbon (DOC) (Wing and Priscu, 1993) led to the hypothesis that phototrophic and heterotrophic microorganisms were present within the lake ice and were metabolically active. Subsequent research showed that adequate liquid water was produced during summer (Fritsen et al., 1998; Adams et al., 1998) to support an active prokaryotic ecosystem within the ice consisting of cyanobacteria and a diversity of bacterial species (e.g., Priscu et al., 1998; Paerl and Priscu, 1998; Pinckney and Paerl, 1996; Gordon et al., 2000).

The phylogenetic diversity of bacteria and cyanobacteria colonizing sediment particles at a depth of 2.5 m in the permanent ice cover of Lake Bonney was characterized by analyses of 16S rRNA genes amplified from environmental DNA (Gordon et al. 1996, 2000). An rRNA gene clone library of 198 clones was made and characterized by sequencing and oligonucleotide probe hybridization. The library was dominated by representatives of the cyanobacteria, proteobacteria, and Planctomycetales, but also contained diverse clones representing the *Acidobacterium* and *Holophaga* division, the Green Non-Sulfur division, and the *Actinobacteria* (Fig. 1). Of the cyanobacterial

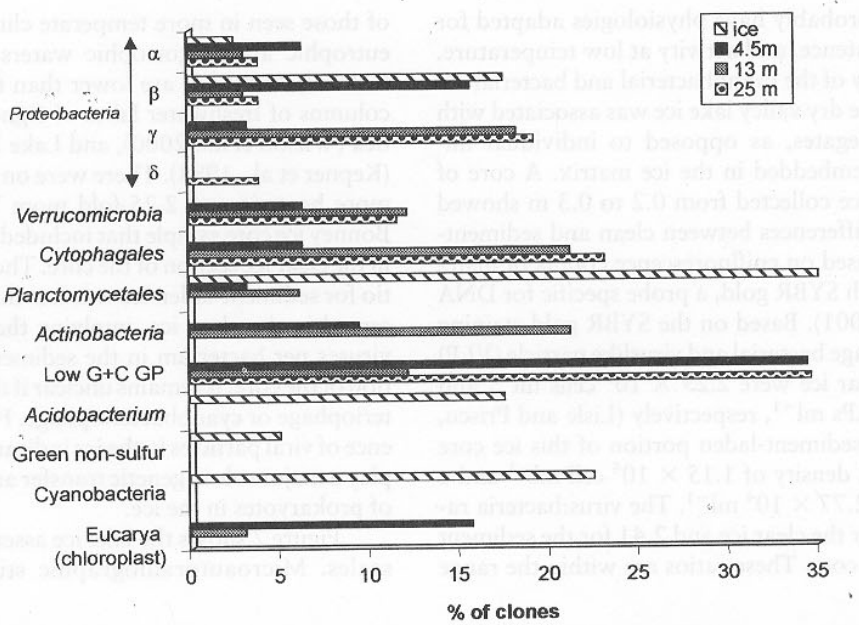

Figure 1. Lake Bonney 16S rDNA summary comparing lake ice sequences with water column sequences. The ice sample was collected about 2 m beneath the surface of the 4-m-thick permanent ice cover; the 4.5- and 13-m samples were from the east lobe, and the 25-m sample was from the west lobe of Lake Bonney. See Priscu *et al.* (1997) for hydrographic characteristics of the water column of these lake basins and Priscu et al. (1998) for details of the ice column. GP, gram positive.

gene clusters characterized, only one was closely (>97% similarity) affiliated with a well-characterized cyanobacterial species, *Chamaesiphon subglobosus*. The remaining cyanobacterial gene clusters were less than 93% similar to any characterized sequences in public databases although they resembled the *Leptolyngbya* sp. and *Phormidium* sp. Oligonucleotide probes made from three lake ice cyanobacterial clusters were used to screen environmental 16S rDNA samples obtained from the terrestrial (soil and stream) environment in the vicinity of Lake Bonney and Lake Fryxell. The probes designed to hybridize to cyanobacterial 16S rRNA genes effectively hybridized to each sample, indicating that the cyanobacterial sequences present in the lake ice of Lake Bonney are also found in terrestrial cyanobacterial mat samples. Molecular characterization (PCR amplification of the *nifH* fragments) of the *nifH* gene (encoding for the highly conserved Fe-protein subunit) of nitrogenase in lake ice sediments from Lake Bonney also demonstrated the presence of a diverse diazotrophic assemblage (Olson et al., 1998). The *nifH* analysis suggested that phototrophic cyanobacteria and heterotrophic microorganisms have the potential to fix atmospheric nitrogen when liquid water is present in the ice cover. The expression of nitrogenase was confirmed by the acetylene reduction assay for nitrogenase activity (Grue et al., 1996; Olson et al., 1998; Paerl and Priscu, 1998). Sequence analysis in concert with physiological data indicates that the cyanobacterial (and bacterial) community within the lake ice is dominated by organisms that did not evolve in the lake ice ecosystem. Instead, the strong katabatic winds common to the region act to disperse microorganisms in the desert environment and provide the biological seed for the lake ice microbial assemblage (Gordon et al., 2000; Priscu et al., 1998). Different prokaryotic sequence data from selected depths within the water column of Lake Bonney further corroborate this contention (Fig. 1), confirming that distinctly different microbial communities exist within the ice and water column. Although cyanobacteria, *Planctomyces*, and *Acidobacteria* dominate the assemblages within lake ice, the most frequently identified water column species are members of the *Verrucomicrobia*, *Cytophagales*, and low-G+C gram-positive bacteria; none of the water column groups were detected in the ice cover. Preliminary analysis of the 16S rDNA sequences obtained at depth in Lake Bonney revealed that ~70% of the clones obtained had high identity with species from marine and lake habitats, with more than half of these sequences being most similar to isolates and clones from polar marine or lake environments (J. P. Zehr and J. C. Priscu, unpublished). The occurrence of related phylotypes from geographically diverse but predominantly cold aquatic environments argues that

these species probably have physiologies adapted for survival, persistence, and activity at low temperature.

A majority of the cyanobacterial and bacterial activity within the dry valley lake ice was associated with sediment aggregates, as opposed to individual microorganisms embedded in the ice matrix. A core of Lake Bonney ice collected from 0.2 to 0.3 m showed considerable differences between clean and sediment-laden layers based on epifluorescence counts of material stained with SYBR gold, a probe specific for DNA (Chen et al., 2001). Based on the SYBR gold-staining study, the average bacterial and viruslike particle (VLP) densities in clear ice were 2.29×10^3 cells ml^{-1} and 1.23×10^4 VLPs ml^{-1}, respectively (Lisle and Priscu, in press). The sediment-laden portion of this ice core had a bacterial density of 1.15×10^4 cell ml^{-1} and a VLP count of 2.77×10^4 ml^{-1}. The virus:bacteria ratio was 5.37 for the clear ice and 2.41 for the sediment sections of the core. These ratios are within the range of those seen in more temperate climates that include eutrophic and oligotrophic waters (Wommack and Colwell, 2000) but are lower than those in the water columns of freshwater lakes of Signey Island, Antarctica (Wilson et al., 2000), and Lake Hoare, Antarctica (Kepner et al., 1998). There were on average 5.02-fold more bacteria and 2.25-fold more VLPs in the Lake Bonney ice core sample that included the sediment than in the clear ice section of the core. The virus:bacteria ratio for sediment-laden ice was less than half of that observed in the clear ice, implying that there are fewer viruses per bacterium in the sediment-containing section of the core. It remains unclear if the VLPs were bacteriophage or cyanobacteriophage. However, the presence of viral particles in the ice indicates that phage may play a major role in genetic transfer and overall survival of prokaryotes in the ice.

Figure 2 shows the lake ice assemblage on several scales. Microautoradiographic studies reveal that

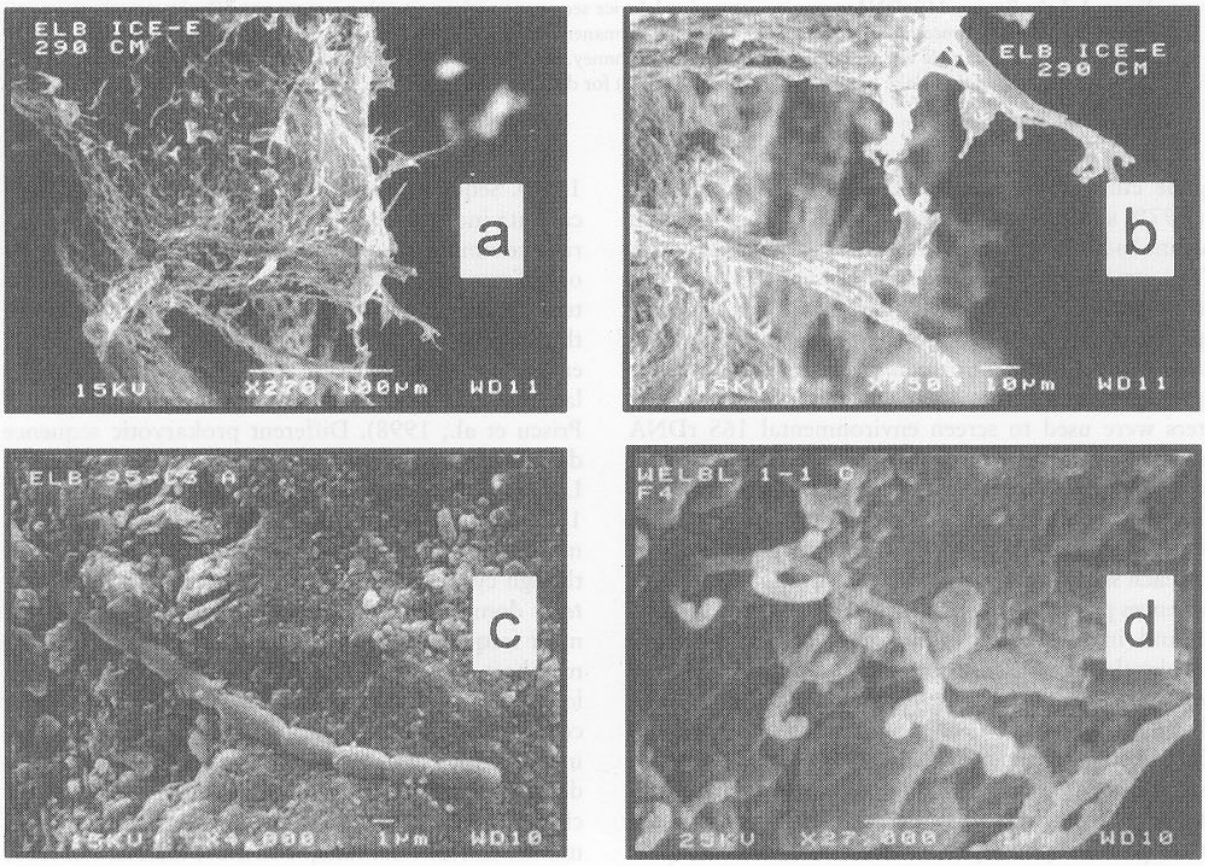

Figure 2. Scanning electron microscope (SEM) images of microbial assemblages collected 2 m beneath the surface of the east lobe Lake Bonney ice cover. (a) and (b) represent low- and high-magnification images of cyanobacterial filaments attached to lithogenic material; (c) a single cyanobacterial filament attached to a surface, (d) small unknown organic filaments attached to a surface. Images were obtained by cryogenic SEM (JEOL-6100 SEM with an Oxford Instruments cryogenic preparation stage) on particles captured by 0.2-µm filtration of melted ice.

both bacterial and cyanobacterial activities were closely associated with sediment particles, corroborating experimental results (Priscu et al., 1998). Microautoradiographs also indicated that virtually all of the incorporation of radiolabeled organic substrates was mediated by nonautofluorescent (nonchlorophyll-containing) bacterial-size rods (0.5 to 1 μm in length) and filaments (0.5 μm wide) closely associated with aggregates, whereas $^{14}CO_2$ incorporation was limited to filamentous cyanobacteria (Paerl and Priscu, 1998). Heterotrophic bacteria were attached to soil particles and associated with cyanobacterial colonies and aggregates. These observations are similar to those reported for temperate and tropical cyanobacteria-dominated systems (Paerl and Pinckney, 1996). Tetrazolium salt reduction assays further revealed that, when melting occurs, localized O_2 consumption associated with aggregates is sufficient to create reduced microzones. These microzones are associated with regions colonized by bacteria and cyanobacteria, suggesting that they may be potential sites for O_2-sensitive processes such as atmospheric nitrogen fixation (Olson et al., 1998; Paerl and Priscu, 1998). Pinkney and Paerl (1996) showed that cyanobacterial and bacterial biomass and activities were heterogeneously distributed among aggregates, promoting the development of O_2 and, possibly, other biogeochemical gradients. Biogeochemical zonation and diffusional O_2 and nutrient concentration gradients likely result from microscale patchiness in microbial metabolic activities (i.e., photosynthesis, respiration). These gradients, in turn, promote metabolic diversity and differential photosynthetic and heterotrophic growth rates.

Phototrophy, heterotrophy, and diazotrophy (N_2 fixation) can occur simultaneously in ice-aggregate microbial communities. Mineralization of particulate organic carbon and particulate organic nitrogen is highly dependent on organic matter availability, the main source being cyanobacterial photosynthesis. Therefore, close spatial proximity of heterotrophs to phototrophs is essential for completion of carbon, nitrogen, and phosphorus cycling. The paucity of higher trophic levels in the ice (e.g., protozoans) magnifies the importance of microbial interactions within the ice assemblage and amplifies the role played by viruses in terms of microbial survival and possibly diversity. Clearly, the spatial and temporal relationships within the ice produce a microbial consortium that is of fundamental importance for initiating, maintaining, and optimizing essential life-sustaining production and nutrient transformation processes (Priscu et al., in press). The close spatial and temporal coupling of metabolites within the microbial consortium appears to be essential for the microbes to survive and replicate in what has been characterized as "the edge of life" (Paerl and Priscu, 1998). Data on microbial activity for the ice assemblage indicate that metabolic complementation among functionally diverse, but structurally simple, prokaryotic consortia along microscale biogeochemical gradients is a unique and effective strategy for meeting the requirements of life in what appears to be an otherwise inhospitable environment.

CRYOCONITE HOLES

Cryoconite holes form as windblown particulates accumulate on the surface of a glacier, are warmed by the Sun, and melt into the ice, producing a cylindrical basin of liquid water (Fig. 3). Cells released from the melted glacial ice and deposited attached to airborne particulates inoculate these environments with viable organisms. Primary production by algae and cyanobacteria supply sufficient reduced carbon and nutrients to support complex microbial and invertebrate communities, and cryoconite hole ecosystems occur globally in Arctic (Gerdel and Drouet, 1960; De Smet and Van Rompu, 1994; Grøngaard et al., 1999; Mueller et al., 2001), Antarctic (Wharton et al., 1981; Christner et al., 2003b), and alpine glaciers (Kohshima, 1989; Takeuchi et al., 2000). During the austral summer in the McMurdo Dry Valleys, the 24 daylight hours and increased temperature enable liquid water to exist on the glacial surface, and the melting process is greatly accelerated in cracks and depressions within the ice that collect heat-absorbing sediments (Wharton et al., 1985). Under these circumstances, aquatic communities based on algal and cyanobacterial photosynthetic primary production develop, but are destined to refreeze, and presumably become inactive, through the cold, dark winter months.

Although dominated by microorganisms, cryoconite holes are one of the few environments in the dry valleys inhabitable by metazoan life, and the resident rotifer, tardigrade, and nematode species have the ability to differentiate under adverse conditions into metabolically dormant forms and, as such, could possibly also survive within a cryoconite sediment that is completely frozen (Spmme, 1996). Every cryoconite hole formed is unique and therefore may support a novel and discrete ecosystem. However, based on results from a phylogenetic survey of a cryoconite hole on the Canada glacier (Christner et al., 2003b), these ecosystems are inhabited by species very similar to those in adjacent microbial mat and lake ice communities in this polar desert environment (Priscu et al., 1998; Gordon et al., 2000). Thus, particulates blown onto the glacier from adja-

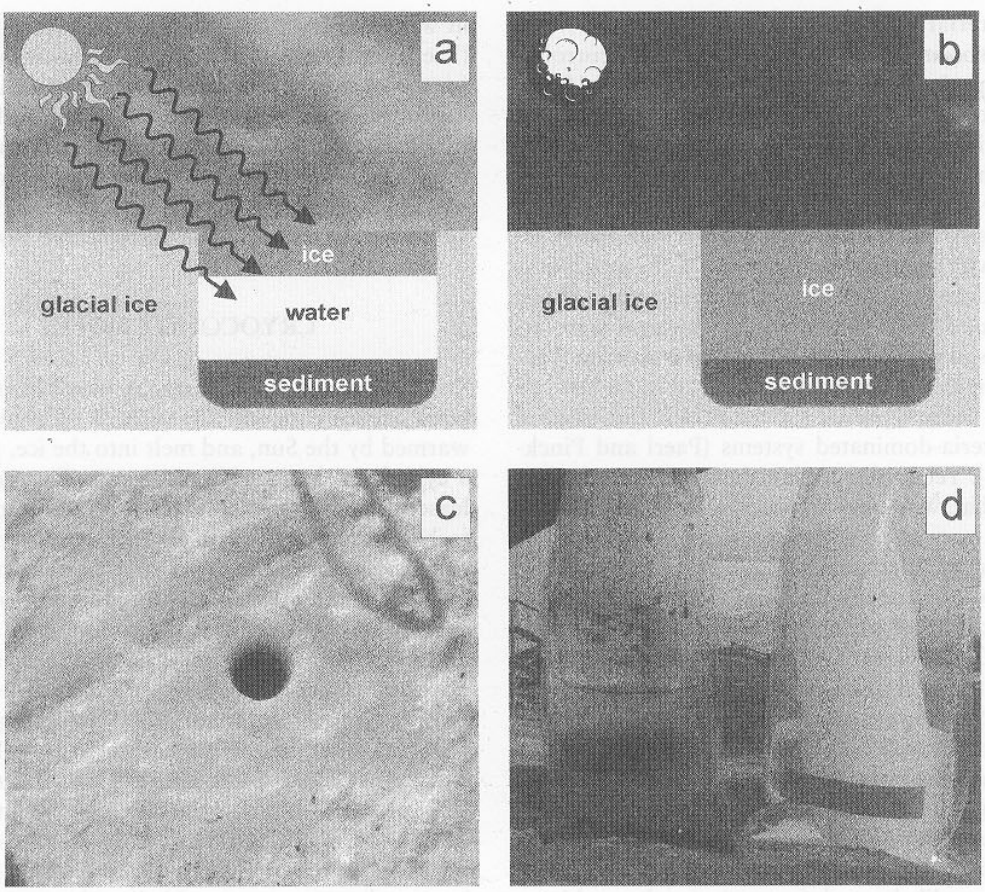

Figure 3. The cryoconite hole environment in the McMurdo Dry Valleys. In summer, sediment collects on glacial surfaces, and exposure to solar irradiation produces (a) melt pools within the ice, which may subsequently freeze on the surface (b) and completely freeze during the winter. The cryoconite hole illustrated in (c) was located on the Canada glacier and was completely frozen when sampled in January 2001. (d) A comparison of cores retrieved from the cryoconite hole (left) with a core from the adjacent glacial ice. Note the dense layer of sediment and organic material present within the bottom 5 cm of the cryoconite hole core.

cent locations are responsible for seeding cryoconite holes with biological material. Although these environments become completely frozen during the Antarctic winter, upon summer warming and glacial melting, the surviving members of these communities might serve in reverse to ensure the reseeding of surrounding environments. The notion that cryoconite holes serve as biological refuges in this very cold, essentially desert environment warrants more detailed investigation.

LIFE IN GLACIAL ICE

Snowfall accumulates into continental ice sheets in the polar regions and globally at high altitudes (Fig. 4). Depending on the topological nature of the accumulation environment, high-elevation ice fields are termed valley or alpine glaciers and termed icecaps when a flat bedrock surface or volcanic crater is completely covered in ice. The expansive icecaps of Greenland and Antarctica cover ~10% of Earth's terrestrial surface with ice and contain ~70% of the fresh water on the planet (Patterson, 1994). Earth's climate is currently in an interglacial stage of a 100,000-year cycle, caused largely by episodic changes in the planet's axial tilt and ellipticity of its orbit around the Sun. During the last glacial maximum 18,000 years ago, sea levels were ≈120 m lower than today and the north polar icecap advanced to cover 5 million square kilometers, blanketing what is now Canada and half of the United States (Hughes, 1998).

Archived chronologically within glacial ice are samples of the atmospheric constituents from different times in the past, including biological material such as insects, plant fragments, seeds, pollen grains,

fungal spores, viruses, and bacteria (Abyzov, 1993; Abyzov et al., 1998; Dancer et al., 1997; Castello et al., 1999; Willerslev et al., 1999; Christner et al., 2000, 2003; Zhang et al., 2001). Ice cores extending thousands of meters below the glacial surface can represent hundreds of thousands of years of snowfall accumulation, and the assemblages of microorganisms immured chronologically within a core are species that were distributed in the atmosphere at different times in history. Studies indicate that the topography, local and global environmental conditions, and proximity of ecosystems contributing biological particles to a particular air mass influence the concentration and diversity of airborne microorganisms (Lighthart and Shaffer, 1995; Giorgio et al., 1996; Fuzzi et al., 1997; Marshall and Chalmers, 1997). Consistent with this, ice core samples from nonpolar, high-altitude glaciers contain a greater number and variety of culturable bacterial species than polar ices, and, similarly, the highest recoveries from polar ice cores were obtained from Antarctic regions adjacent to exposed soils and rock surfaces of the McMurdo Dry Valleys complex (Christner et al., 2000). Hence, increased microbial deposition occurs in glaciers contiguous to environments that supply airborne rock grains and soils, which presumably serve to transport and protect attached microorganisms.

Aerosolized microorganisms can travel large distances on atmospheric currents, often in a viable but dormant state. Remarkably, some air conditions actually provide a medium for growth, and microbial metabolism has been detected in fog particles (Fuzzi et al., 1997) and supercooled clouds (Sattler et al., 2001). For an airborne microorganism deposited in glacial ice to retain viability, the stress associated with desiccation, solar irradiation, freezing, an extended period of no growth, and subsequent thawing must not result in a lethal level of unrepairable cellular damage. It is therefore not surprising that many of the species that are isolated form spores, structures known to confer resistance to environmental abuses. Many also have thick cell walls or polysaccharide capsules and resist repeated cycles of freezing and thawing. Regardless of the ice cores' geographical source, related but not identical species are frequently recovered. Interestingly, members of the bacterial genera *Sphingomonas, Acinetobacter,* and *Arthrobacter* are commonly isolated from glacial samples (Christner

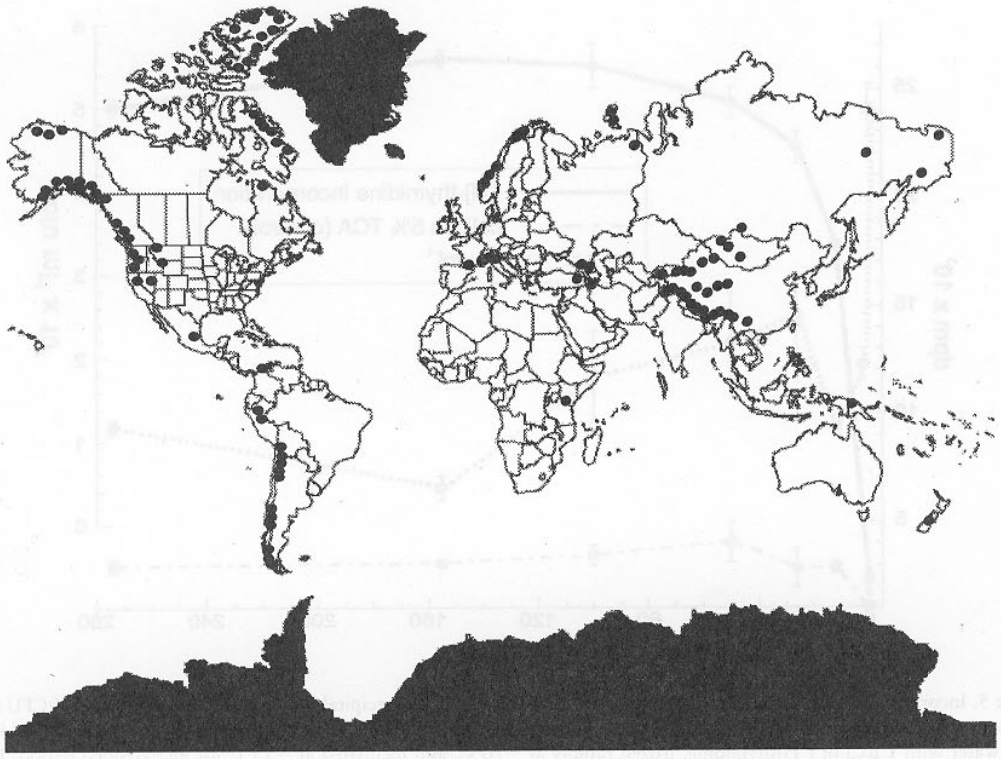

Figure 4. Global locations of existing glacial ice sheets and caps (denoted by shading). At each geographical location, the nearest terrestrial or marine ecosystem that would most likely contribute the majority of airborne particles are very different.

et al., 2000, 2001, 2003a), and these are also the most frequently isolated genera in enrichment surveys of terrestrial subsurface environments (Balkwill et al., 1997). As such, these genera would appear to contain species that can survive for extended times under low-nutrient, nongrowth conditions, and similar survival strategies may be in effect in deep ice and subsurface situations.

Viable cells and nucleic acids remain preserved for hundreds of thousands of years in glacial ice (Abyzov, 1993; Christner et al., 2003a). Although many glacial isolates appear to possess features that might enhance their survival while dormant (i.e., ability to form spores), the thermodynamic reality is that, in the absence of metabolic activity, cells must incur a significant amount of macromolecular damage over such long periods of time. Temperature and the hydration of nucleic acids and protein strongly influence the rate of depurination and L-amino acid racemization, respectively (Lindahl, 1993; Bada et al., 1994). Amino acids in amber have retarded racemization rates, with the observed stereochemical preservation attributed to the anhydrous nature of amber (Poinar et al., 1996), and this could also pertain to ice. It is also possible that entrapped microbes carry out a slow rate of metabolism which allows repair of macromolecular damage, but not growth. Thin veins of liquid water between ice crystals could potentially provide a microbial habitat within apparently solid ice (Mader et al., 1992a,b; Price, 2000). Studies of permafrost (Rivkina et al., 2000), surface snow (Carpenter et al., 2000), and frozen bacterial suspensions (Fig. 5) have demonstrated low levels of metabolic activity at subzero temperatures. Based on the minimum required input to avoid microbial carbon loss, Price (2000) calculated that the vein environment contains sufficient carbon and nutrients to support a small population of cells (10 to 10^2 cells ml^{-1}) for hundreds of thousands of years. Indirect evidence for microbial activity in glacial ice was obtained when analysis of the air bubbles in cores from Vostok Station, Antarctica, and Sajama, Bolivia, revealed isotopic fractionation profiles consistent with in situ microbiological production of nitrous oxide and methane, respectively (Sowers, 2001; T. Sowers, personal communication). Geochemical anomalies attributed to microbial activity in Greenland ice have also been reported (Souchez et al., 1995, 1998), and this issue must now be experimentally addressed, particularly with respect to the interpretation of paleoclimate records obtained from ice cores.

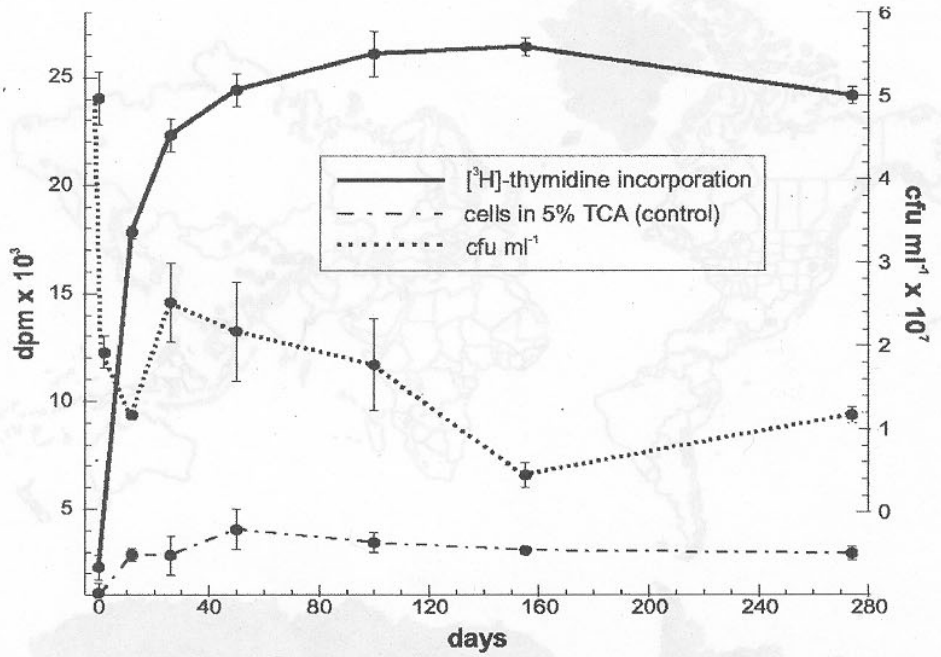

Figure 5. Incorporation of [^{3}H]thymidine into trichloroacetic acid (TCA)-precipitable material and the number of CFU mL^{-1} for the glacial isolate *Psychrobacter* sp. Trans 1 after 9 months at −15°C. Cells in logarithmic growth were suspended in distilled water with 1 μCi of [^{3}H]thymidine, frozen rapidly at −70°C, and incubated at −15°C for an extended period. Under these circumstances, cells were able to conduct a low level of macromolecular synthesis, but this activity was not sufficient for reproductive growth. For more details, see Christner 2002.

SUBGLACIAL LAKES

Much attention is currently focused on the exciting possibility that the subglacial environments of Antarctica may harbor microbial ecosystems under thousands of kilometers of ice, isolated from the atmosphere for as long as the continent has been glaciated (20 to 25 million years, [Naish et al., 2001]). The discovery of more than 70 subglacial lakes in central Antarctica during the early 1970s (Siegert et al. 1996; Siegert, 2000) went relatively unnoticed by the biological scientific community; however, curiosity about the nature of these environments has recently intensified as a result of the discovery of other large subglacial lakes (McKay et al., in press; Tobacco et al., 1998; Tikku et al., 2002) and increasing international interest in the largest subglacial lake, Lake Vostok. The freshwater in Lake Vostok originates from the overlying ice sheet, which melts near the shoreline of the lake and at the ice-water interface in the north and is thought to have a relatively high dissolved oxygen content supplied from air bubbles released from overlying glacial ice (Lipenkov and Barkov, 1998; Lipenkov and Istomin, 2001; Lipenkov et al. 2000). Lake water refreezes (accretes) at the base of the ice sheet in the central and southern regions, removing water from the lake (Kapitsa et al., 1996; Jouzel et al., 1999; Siegert et al., 2001; Bell et al., 2002). Hence, constituents in the accretion ice should reflect those in the actual lake water in a proportion equal to the partitioning that occurs when the water freezes (Priscu et al., 1999; Karl et al., 1999, Siegert et al., 2001). Vostok ice coring reached a record depth of 3,623 m in 1998, but due to concerns regarding contamination, drilling stopped at ~120 m above the lake-ice interface; the deepest part of the core recovered 150 m of accretion ice (Petit et al., 1999). The presence of microorganisms within Lake Vostok accretion ice has now been confirmed independently by at least three laboratories (Priscu et al., 1999b; Karl et al., 1999; Christner et al., 2001). Molecular profiling of accreted ice microbes using 16S rDNA techniques (Priscu et al., 1999b; Christner et al., 2001) showed close agreement with present-day surface microbiota. Phylotypes have mapped closely to extant members of the *Alpha-* and *Betaproteobacteria* and to *Actinomycetes* (the latter also isolated in Vostok glacial ice [Abyzov et al., 1998]). If the accreted ice microbes are representative of the lake microbiota, this would imply that microbes within Lake Vostok do not represent an evolutionarily distinct subglacial biota (Siegert et al., 2003). The time scale of isolation within Lake Vostok (~20 to 25 million years) is not long in terms of prokaryotic evolution compared with its 3.7×10^9-year history on Earth, and studies of species divergence of other prokaryotes have shown that species-level divergence may take ~100 million years (Lawrence and Ochman, 1998). However, other mechanisms of genetic change (such as recombination and mutator genes) could allow more rapid alteration of organism phenotype allowing for adaptation to conditions within Lake Vostok (Page and Holmes, 1998). An alternative scenario is that glacial meltwater entering the lake forms a lens overlying the Vostok water column. If so, the microbes discovered within accretion ice would likely have spent little time within the actual lake water itself (few, if any, cell divisions occurring) before being frozen within the accretion ice. The microbes within the main body of the lake below such a freshwater lens may have originated primarily from basal sediments and rocks, and if so, their period of isolation may be adequate for significant evolutionary divergence, particularly given the potential selection pressures that exist within the subglacial environment.

A recent report on microbial diversity in Lake Vostok accretion ice, using PCR-based analyses of 16S rDNA in accretion ice (Bulat et al., 2002), has revealed three phylotypes closely related to DNA signatures representative of thermophiles. One of them is a known extant chemolithoautotroph identified previously in hot springs and capable of obtaining energy by oxidizing H_2S at reduced O_2 tension. Two other taxa are not identified in the current databases, but showed relatedness to bacteria associated with hydrothermal vents and associated surface sediments. Evidence for the presence of thermophiles is supported by the recent interpretation by French scientists of He^3/He^4 data from accretion ice (Petit et al., 2002). This interpretation now implies that there may be extensive faulting beneath Lake Vostok resulting in hydrothermal activity in the southern part of the lake. If this emerging picture is correct, Lake Vostok could harbor a unique assemblage of organisms fueled by chemical energy. Although it seems inevitable that viable microorganisms from the overlying glacial ice and in sediment scoured from bedrock adjacent to the lake are regularly seeded into the lake, the question remains whether these or preexisting microorganisms have established a flourishing community within Lake Vostok. If a microbial ecosystem were found to exist within the water or sediment of this subsurface environment, it would represent one of the most extreme and unusual environments on Earth.

Abyzov et al. (1998) measured bacterial cell densities ranging from 1.2×10^3 to 8.3×10^3 in Vostok glacial ice between 1,665 and 2,750 m and showed that density was positively correlated with atmospheric microparticles within the ice. The highest cell and atmospheric particle densities occurred during a glacial period indicating that paleoclimate

Table 1. Summary of the bacterial cell number and organic carbon contribution from Antarctic subglacial lakes and the Antarctic and Greenland ice sheets[a]

Parameter	Antarctica			Greenland ice sheet	Both poles, lakes + ice sheet	Global		
	Lakes	Ice sheet	Total			Fresh waters	Open ocean	Soils
Cell number ($\times 10^{25}$)	1.20	8.84	10.04	0.77	10.81	13.1	10,100	26,000
Cell-C (Pg) ($\times 10^{-3}$)	0.33	2.44	2.77	0.21	2.99	3.63	2,790	26,000
DOC (Pg)	0.01	3.31	3.32	0.29	3.61	NA	NA	NA
Cell-C+DOC (Pg)	0.02	3.32	3.34	0.29	3.62	NA	NA	NA

[a] Carbon concentrations are in petagrams (Pg = 10^{15} g). Ice sheet DOC was estimated assuming an ice concentration of 0.11 mg liter^{-1}; cell number and cell-C for the Greenland ice sheet assume bacterial densities similar to those measured in the Antarctic ice sheet. Global estimates for cell number and cell carbon (cell-C) are from Whitman et al. (1998). Freshwaters represent all surface rivers and lakes, excluding subglacial lakes. NA, not computed.

has a major role in the distribution and perhaps type of microorganisms found in the ice. Priscu et al. (1999b) used epifluorescence microscopy of DNA-stained cells and scanning electron microscopy to measure bacterial cell densities of 2.8×10^3 and 3.6×10^4, respectively, in Vostok accretion ice (3590 m). Priscu et al. further reported DOC concentrations in core 3590 of 0.51 mg l^{-1}. Based on these values and estimates of partitioning coefficients for the water to ice phase change, they estimated that the water in Lake Vostok had bacterial cell concentrations of 10^5 to 10^6 ml^{-1} and a DOC concentration of 1.2 mg l^{-1}. Using data on the volume of the Antarctic ice sheet and subglacial lakes (Siegert, 2000) in concert with published bacterial volume-to-carbon conversion factors (Riemann and Spndergaard, 1986), we estimated the cell number and carbon content within the Antarctic ice sheet and Antarctic subglacial lakes (Table 1). Our estimates assume that concentrations of bacterial cells and DOC in all subglacial lakes are similar to those estimated for Lake Vostok. Based on these calculations, subglacial lakes contain about 12% of the total cell number and cell carbon with respect to the pools associated with the Antarctic continent (e.g., subglacial lakes plus ice sheet). The number of prokaryotic cells we estimate for subglacial lakes plus the ice sheet (10.04×10^{25} cells) is about 50-fold higher than that estimated for prokaryotic cells in Antarctic sea ice (2.2×10^{24} cells) and about 25-fold higher than sea ice in both polar regions combined (4×10^{24} cells) (Whitman et al., 1998). The prokaryotic carbon content of subglacial lakes plus the ice sheet is about 76% of that estimated for all of Earth's liquid surface fresh waters (rivers plus lakes, excluding Antarctic subglacial lakes) but more than 3 orders of magnitude less than that in the open ocean and soils. The number of cells we estimate for Antarctic subglacial lakes alone (1.20×10^{25}) approaches that reported by Whitman et al. (1998) for the Earth's surface freshwater lakes and rivers combined (13.1×10^{25}). To our knowledge, no data on cell density have been published for the Greenland ice sheet. Assuming that prokaryotic cell densities in the Greenland ice sheet are the same as those measured in the Vostok ice core, we estimate that Greenland adds about 8% to the carbon pools we estimated for the Antarctic ice sheet. Our seminal estimates of the number of prokaryotes and organic carbon associated with polar ice and Antarctic subglacial lakes are clearly tentative and should be refined once additional data become available. These calculations, however, do imply that polar ice, particularly Antarctic ice, contains an organic carbon reservoir that should be considered when addressing issues concerning global carbon dynamics.

EVIDENCE FOR COLD-ADAPTED MICROBIAL SPECIES

Molecular-based approaches to microbial ecology yield data that measure the natural evolutionary relationships between microorganisms. As such, a phylogenetic comparison of the species inhabiting similar environments provides a way to examine biogeographical relationships—an essential prerequisite to determine global biodiversity and resolve the ecological role of species distributed throughout the biosphere. A number of studies have examined bacterial and archaeal biogeography in soil, microbial mat, hot spring, hydrothermal vent, oil reservoir, and polar seawater and ice environments (Stetter et al., 1993; Garcia-Pichel et al., 1996; Fulthorpe et al., 1998; Staley and Gosink, 1999; Reysenbach and Shock, 2002; Van Dover et al., 2001; Hollibaugh et al., 2002). Cosmopolitan microorganisms are found globally

(Stetter et al., 1993; Garcia-Pichel et al., 1996; Fulthorpe et al., 1998; Hollibaugh et al., 2002), and Staley and Gosink (1999) hypothesized that endemism results from the inability of certain species to survive dissemination through air or water to other locations suitable for colonization.

Figure 6 illustrates the phylogenetic relatedness, based on 16S rDNA identity, between bacteria recovered in our laboratories and by others (Benson et al., 2000) from Antarctica and permanently cold nonpolar locales. As indicated, these psychrophilic and psychrotrophic isolates originate from locations ranging from aquatic and marine ecosystems to terrestrial soils and glacial ice, with little in common between these environments except that all are permanently cold or frozen. The isolation of related species from such diverse frozen environments argues that clades in these bacterial genera evolved under cold circumstances and likely possess similar strategies to survive freezing and remain active at low temperature. Although not possible through analysis of a single gene, a polyphasic approach could reveal patterns of conserved inheritance and divergence from a common ancestor or identify parallel evolutionary pathways. Such information, coupled with a dedicated effort to further investigate microbial diversity within the planet's frozen realms, will provide the perspective necessary to understand the evolution and ecological impacts of microbial ecosystems residing within Earth's icy biosphere.

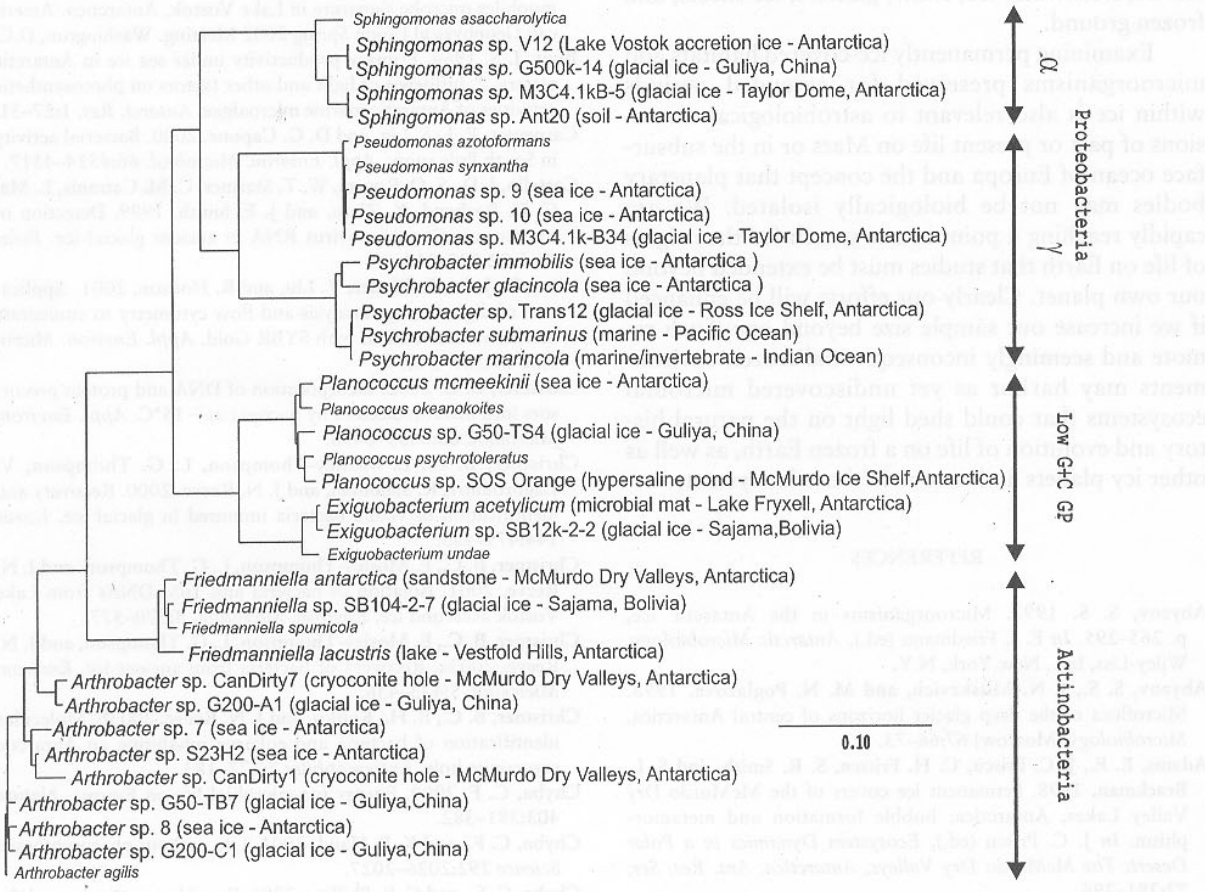

Figure 6. Phylogenetic analysis of bacteria obtained in microbiological surveys of permanently cold and frozen environments. Isolates from cold habitats are shown in bold, followed by the source environment and geographical location. The 16S rDNA sequences corresponding to nucleotides 27-1492 of the *Escherichia coli* 16S rDNA were aligned based on secondary structure and used to construct this neighbor-joining tree. The scale bar represents 0.1 fixed substitutions per nucleotide position. GP, gram positive.

CONCLUDING REMARKS

From a microbial perspective, our planet's zone of habitation (the biosphere) extends from high into the atmosphere to the inner depths of the Earth, where temperatures rise with depth to exceed those assumed possible for known carbon-based life, to the bottom of ice sheets where temperatures rarely exceed 0°C. Based on information gathered over the past 5 years, it is clear that the role of permanently cold ecosystems in global ecology must be reassessed and included in formal definitions of Earth's biosphere. As such we propose that the Earth's cryosphere and associated sub-ice lakes should be included as biospheric components of our planet. Cryosphere is defined here as that portion of the Earth's surface where water is in a solid form as snow or ice. Water in its solid form includes sea ice, freshwater ice, snow, glaciers, ice sheets, and frozen ground.

Examining permanently ice-covered habitats and microorganisms preserved for extended periods within ice is also relevant to astrobiological discussions of past or present life on Mars or in the subsurface ocean of Europa and the concept that planetary bodies may not be biologically isolated. We are rapidly reaching a point in our search for the origins of life on Earth that studies must be extended beyond our own planet. Clearly our efforts will be enhanced if we increase our sample size beyond one. Such remote and seemingly inconsequential frozen environments may harbor as yet undiscovered microbial ecosystems that could shed light on the natural history and evolution of life on a frozen Earth, as well as other icy planets and moons in the solar system.

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