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Classification of Bacteria from Polar and Nonpolar Glacial Ice

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SNOWFALL ACCUMULATES as glacial ice at both poles and globally at high altitudes in nonpolar regions. Archived chronologically within these glaciers are samples of the atmospheric constituents at the time of snow deposition, including particulates of inorganic and biological origin deposited originally on the surface of the snow, often by attachment to snowflakes. Studies of ice cores have established past climate changes and geological events, both globally and regionally, but rarely have these results been correlated with the insects, plant fragments, seeds, pollen grains, fungal spores, and bacteria, that also are present, and very few attempts have been made to determine the diversity and longevity of viable species entombed in such glacial ice. Fungi, algae, protists, bacteria, and viruses have been detected and recovered from polar ice cores (1, 2, 3, 10, 15, 41), but there are very few similar reports describing the microorganisms preserved in nonpolar glacial ice of different age and from different locations. Fortunately, for such studies, we have access to ice cores archived at the Byrd Polar Research Center (BPRC) at the Ohio State University. These ice cores have been collected over many years from globally distributed sites, and many have already been subjected to extensive physical and chemical analyses. These, therefore, provide the opportunity to isolate and to characterize microorganisms from glacial ice formed at defined dates, under known climate conditions, at geographically very different locations (Figure 15.1). To avoid problems of surface contamination, we constructed an ice core sampling system that melts the ice and collects only the resulting interior core meltwater. Here we review the results of bacterial isolations from meltwater generated using this system from the interiors of nonpolar and polar glacial ice cores of different vintage, and from Lake Vostok accretion ice (12,14).

Ice Core Sampling

Ice core exteriors are contaminated during drilling and transport, and a sampling system was designed and constructed to melt ice and collect the resulting

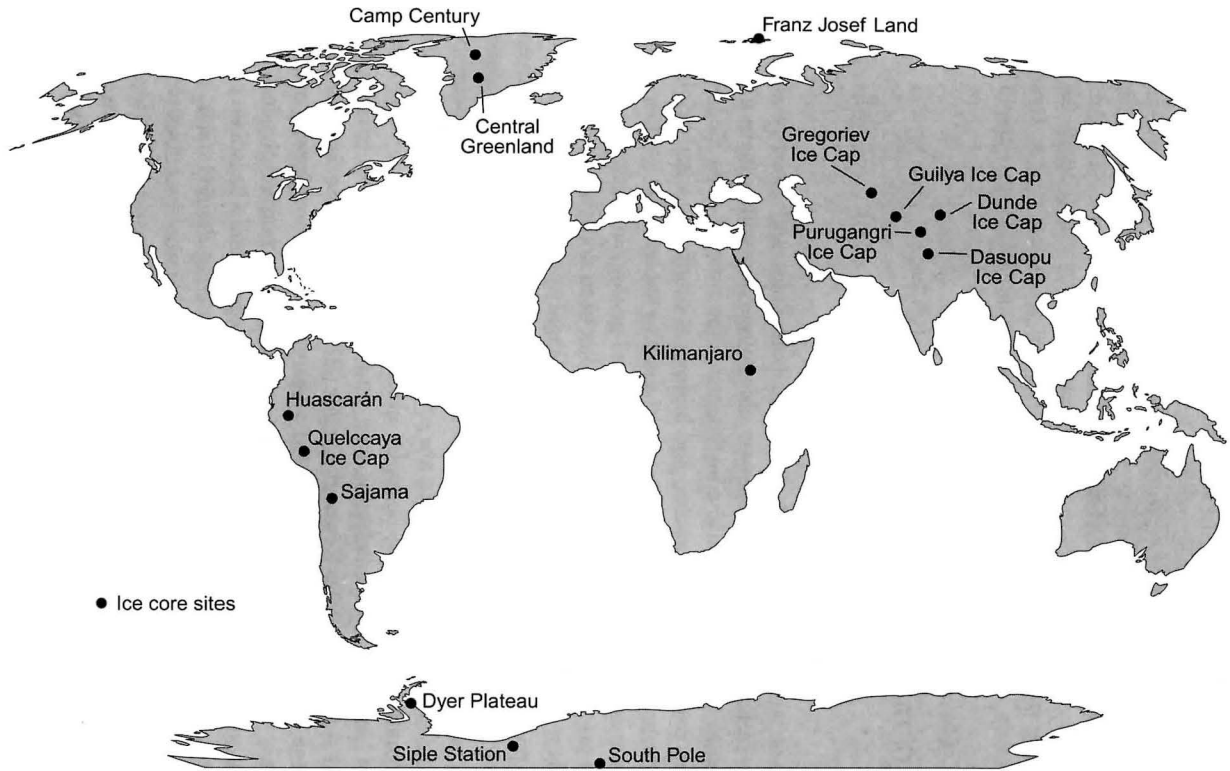

FIGURE 15.1. Sampling sites and ice cores available for study at the Byrd Polar Research Center (BPRC). To date, bacteria have been isolated from ice cores sampled from glaciers at both poles, in the mountain ranges on the subtropical Tibetan plateau, and in the tropical Bolivian Andes. In each case, the nearest major ecosystem, and therefore most likely origin of airborne particulates, is very different.

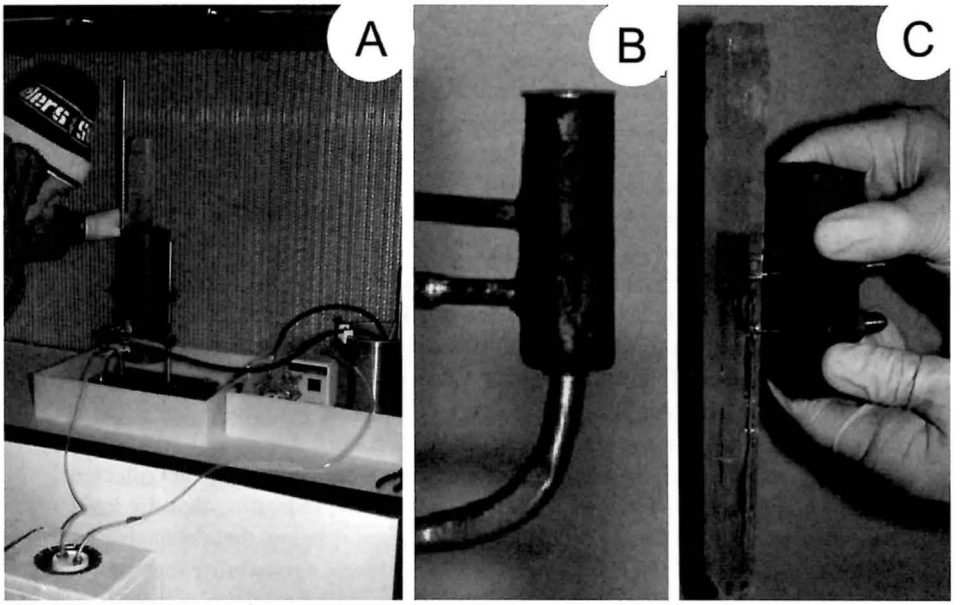

FIGURE 15.2. The ice core sampling system. (A) The sampling system is assembled completely inside a laminar flow hood that is housed within a −10 °C walk-in freezer. All components of the system are autoclaved, dried, and exposed to ethylene oxide for 12 hours before use. An ice core is positioned vertically in the sampler with the cut end of the core contacting (B) the heated sampler head which melts upward (C) through the core and collects the resulting meltwater. In (C), the sampler head is shown disassembled from the main unit to illustrate its movement through the interior of the ice core.

meltwater aseptically only from the inside of an ice core (Figure 15.2) . A thin section is first cut from one end of the core using a dedicated dust-free bandsaw, and the newly exposed flat surface is immersed for 2 minutes in 95% ethanol. Exposure to ethanol does not cause the ice core to fracture and, in reconstruction experiments, such an ethanol treatment effectively killed all *Serratia marcesens* cells that were intentionally swabbed onto the saw blade and onto the resulting cut surface of the ice core before the ethanol treatment. However, this treatment may not kill all bacterial endospores, and it certainly would not destroy nucleic acids. Therefore, to monitor for such contamination, the cut surface of each ice core is swabbed after the ethanol treatment before initiating melting. These swabs are used to inoculate growth media and are evaluated for the presence of DNA by polymerase chain reaction (PCR) amplifications using universal 16S rDNA amplification primers. Immediately after the exposure to ethanol, the ice core is positioned vertically in the sampling system with the ethanol-washed surface placed directly in contact with the

sampling unit. The sampling unit is heated internally, and as it melts the ice, it moves upward through the ice core. The water generated passes through an orifice in the center of the sampling unit, and is collected aseptically in sterile containers positioned outside the sampling system (Figure 15.2).

During the course of this study, growth occurred only once from a swab-inoculated culture and this isolate had a 16S rDNA sequence consistent with classification as an endospore-forming *Bacillus subtilis* species. If this same species had subsequently been isolated from the interior of the core, it obviously would have been suspected as a potentially introduced contaminant. Taq polymerase preparations from several manufacturers were examined for DNA contamination using a nested PCR approach with the universal primers 27F and 1492R and 515F and 1392R (25). Consistent results were obtained with the LD (low DNA) Amplitaq Gold DNA polymerase (Perkin-Elmer Biosystems), which the manufacturer claims has < 10 copies of bacterial 16S rDNA per 2.5 U of enzyme. No amplicons were generated when material collected on the ethanol-washed surface was used in the PCR, indicating that the level of contamination remaining on the ice core surface is below that detectable by standard PCR procedures. Although there was always a possibility that microbial or nucleic acid contamination would elude decontamination procedures, this concern was addressed by employing quality control measures and drawing conclusions from results obtained from more than one ice core sample.

Bacteria Recovered from Glacial Ice

The numbers and identities of bacteria that form colonies when meltwater is plated directly on solid media have been determined in ice from Sajama (Bolivia), Guliya (China), Greenland, and Antarctica. A range of different media was used during the course of this study, and the largest numbers of colonies were routinely observed after aerobic incubation at 15 to 22 °C on media containing low levels of nutrients, such as R2A and 1% nutrient and tryptic soy agar (Difco). In general, meltwaters from nonpolar, low-latitude, high-altitude glaciers contain greater numbers, as well as more diversity of colony-forming bacteria than meltwaters from polar ice cores. For example, 180 colony-forming units per ml (cfu/ml) were present in meltwater from a 200-year-old sample of Guliya ice, whereas water from a ca. 1800-year-old sample of polar ice from Taylor Dome (Antarctica) contained only ca. 10 cfu/ml. Even fewer cfus were present in meltwater from ice of a similar vintage from the Antarctic Peninsula and from the Summit and Dye 2 sites in Greenland. It is important to note that differences in the amount of annual snowfall and in the subsequent rates of compression mean that equal volumes of meltwater from different cores do not necessarily represent equivalent time periods of microbial deposition. However, these results are consistent with those of Dancer et al. (15) who recovered < 5

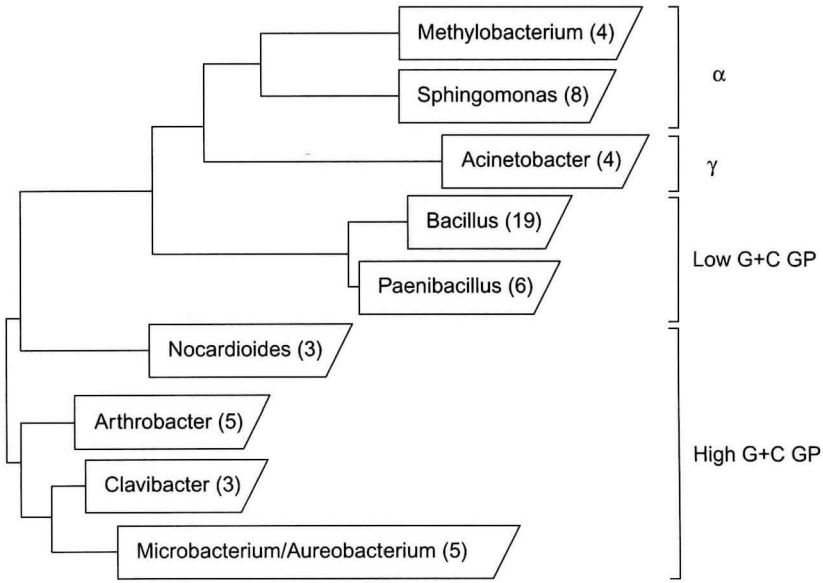

FIGURE 15.3. Bacterial genera represented most frequently by ice core isolates. The number of isolates from both polar and nonpolar ice cores, obtained from each of the bacterial genera shown, is listed in parentheses. The phylogenetic relationships illustrated are based on 16S rDNA sequences. They are not drawn to scale.

cfu/ml from glacial ice from the Canadian high Arctic after enrichment for coliform bacteria, and other reports of recovering even fewer bacteria (< 1 cfu/ml) from meltwaters from polar ice (2, 19). Logically, these differences arise because nonpolar glaciers are closer to major sources of airborne microorganisms such as exposed soils and tropical and subtropical ecosystems. Consistent with this, meltwater from ice from a Taylor Dome site located at the head of the Taylor Valley in the Dry Valley complex of Antarctica contained relatively larger numbers of culturable bacteria (ca. 10 cfu/ml), and microbiological surveys have documented the abundance of bacteria, fungi, and algae in this area despite the very dry and cold climate (6, 30).

Based on their small-subunit ribosomal RNA-encoding sequences (16S rDNAs), most of the ice core isolates are members of the nonsporulating Gram-positive, spore-forming *Bacillus, Paenibacillus*, and *Actinobacteria*, and α- and γ-proteobacterial lineages (Figure 15.3). Many form colored colonies, consistent with pigment production providing protection from solar irradiation during airborne transport and subsequent exposure on the glacier surface. Not surprisingly, a number of the isolates are related to species that have life cycles with radiation- and desiccation-resistant resting stages. Endospore-forming relatives of the genera *Bacillus* and *Paenibacillus* were commonly isolated

from nonpolar glacial ices, presumably due to their close proximity to soil ecosystems, and similar but not identical species of *Sphingomonas*, *Methylobacterium*, *Acinetobacter*, and *Arthrobacter* also were ubiquitous and recovered from both polar and nonpolar locations. While most of the isolates are similar to species frequently found in environmental surveys from around the world, some of the isolates have 16S rDNA sequences most closely related to species recovered previously from Antarctic lake mats (see ref. 6 and chapter 3, this volume), sea ice (see refs. 5, 18, 22 and chapter 4, this volume), and other cold environments (4, 32). The isolation of related microbes from many geographically diverse but predominantly frozen environments argues strongly that these species probably have features that confer resistance to freezing and survival under frozen conditions.

Isolation of Bacteria from Very Old Glacial Ice

An ice core that extends over 300 m below the surface (mbs) to the underlying bedrock was obtained from the Guliya ice cap in Tibet (Figure 15.1), and based on the abundance of ^{36}Cl (half-life = 301,000 years) the ice at the bottom of this core is > 500,000 years old (39). This is the oldest glacial ice recovered to date and provides an opportunity to evaluate microbial survival in ice over an extended period of time (13). Aliquots of meltwater from this ice core from 296 mbs were inoculated into a variety of growth media and, after 30 to 60 days of aerobic incubation at 4 °C, growth was observed in very dilute nutrient and tryptic soy broths. These media were used at 1% of the concentration recommended by the manufacturer (Difco, Inc.). Despite the long period needed for initial growth, and the primary enrichment cultures being grown under oligotrophic conditions at 4 °C, isolates were subsequently obtained from these cultures that grew and formed colonies in two to seven days on nutrient-rich media at 25 °C. Long-dormant cells must eliminate toxic metabolites, such as hydrogen peroxide, superoxide, and free radicals, and repair macromolecular damage that has accumulated before they can grow and divide successfully (16). The results with the very old Guliya ice are consistent with this hypothesis, and indicate that successful recovery is facilitated by providing only a very low level of nutrients initially, sufficient for repair but insufficient to elicit an instant attempt at growth.

Fourteen 16S rDNA sequences, corresponding to nucleotides 27 through 1992 of the *Escherichia coli* 16S rDNA sequence, have been determined from isolates from the very old Guliya ice (Figure 15.4). Based on these data, most of these belong to the same bacterial lineages as the isolates obtained from more recent polar and nonpolar glacial ices, and ca. 50% are members of genera that form endospores known to facilitate long-term survival under non-growth conditions (8, 40). Light microscopy has revealed that some also have

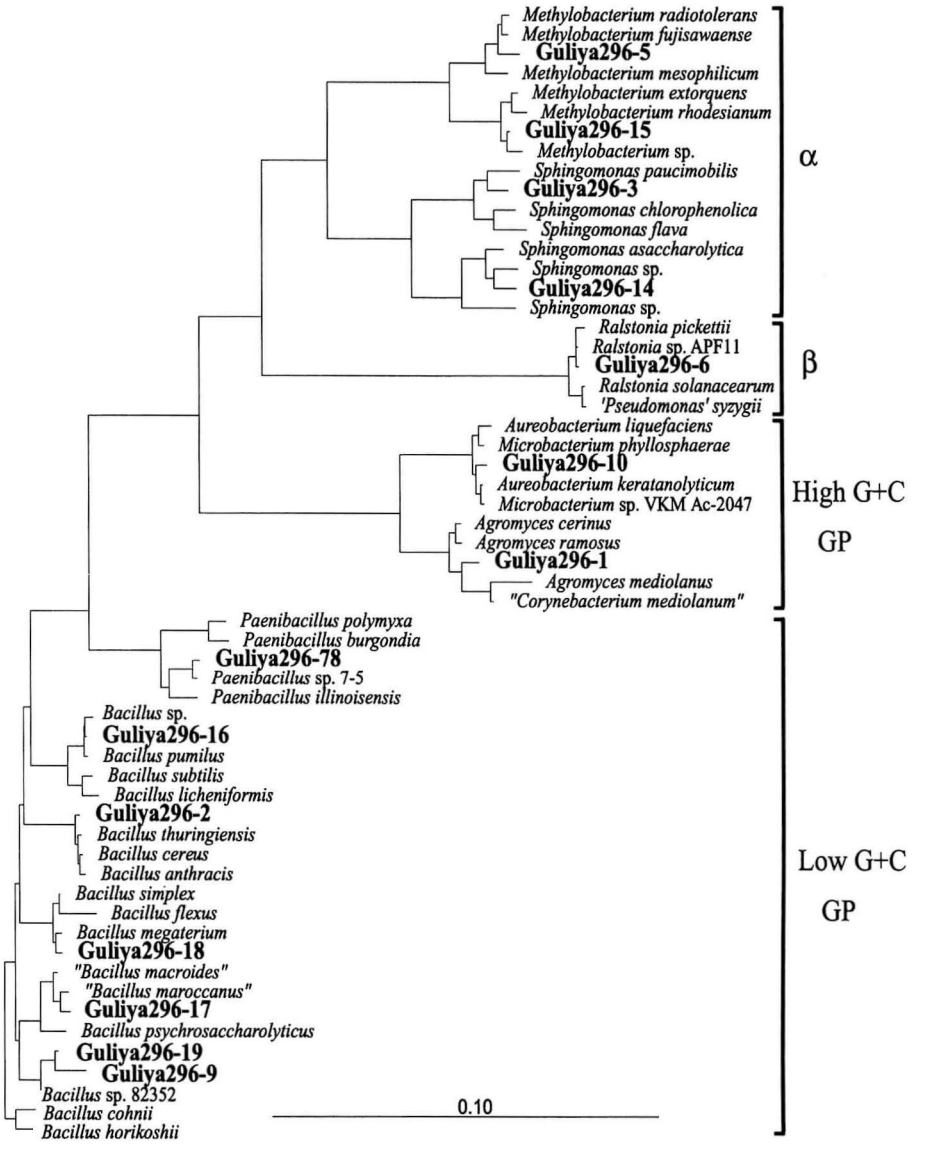

FIGURE 15.4. Phylogenetic position of fourteen bacterial isolates from ice > 500,000 years old from 296 m below surface of the Guliya ice cap. 16S rDNA sequences (ca. 1400 nucleotides) were obtained from the cells from a single colony of each isolate. They were aligned based on secondary structures using the ARB software package (25) and a best fit neighbor-joining tree was constructed. Evolutionary distance is defined as the number of fixed nucleotide changes per position.

thick cell walls and form polysaccharide capsules that presumably also contribute to survival through the physical stresses imposed by freezing, compaction pressure, and thawing (17).

Isolation of Bacteria from Lake Vostok Accretion Ice

More than seventy subglacial lakes have been discovered in Antarctica. The largest, Lake Vostok, has been covered by a layer of glacial ice and isolated from direct surface input for at least 420,000 years (27). Glacial ice melts into Lake Vostok at the northern ice-water interface, and water from Lake Vostok freezes and accumulates as accretion ice directly below the glacial ice over the central and southern regions (21, 23, 33). It seems very likely that viable bacteria are seeded into Lake Vostok as glacial ice melts into the lake. However, whether an active microbial community is established within Lake Vostok remains uncertain, as concerns for contamination have resulted in a moratorium on direct sampling of Lake Vostok water. Ice core drilling also was terminated above the ice-water interface although an ice core was retrieved in which the bottom ca. 150 m are accretion ice and therefore represent a sample of Lake Vostok water. Microbial cells in meltwater from sections of this accretion ice core that originated 3590 and 3603 m below the surface (mbs) have been detected by epifluorescence and scanning electron microscopy (24, 29), and seven bacterial 16S rDNA sequences were amplified from the 3590 meltwater that originated from α- and β-proteobacteria, and from an actinobacteria (29). Evidence for respiration was also obtained by measuring ^{14}C-CO_2 release during incubation at 3 °C and 23 °C after the addition of ^{14}C-acetate or ^{14}C-glucose to meltwater from the 3603 section (24). A section of this core from 3591.965 to 3592.445 mbs, designated as core section 3593, was obtained from the National Ice Core Laboratory (Denver, Colorado), and has been subjected to microbiological investigation (12).

Scanning electron microscopy of materials filtered from core 3593 meltwater revealed the presence of particulates with size and morphology consistent with bacterial cells (Figure 15.5), and four different single-colony isolates were obtained from enrichment cultures inoculated with core 3593 meltwater. Based on their 16S rDNA sequences, these isolates are related to established species of *Brachybacterium, Sphingomonas, Paenibacillus*, and *Methylobacterium* (Figure 15.6, also see photos in chapters 6 and 16). Six bacterial 16S rDNAs also were amplified from core 3593 meltwater with sequences, indicating that they originated from five different bacterial lines of descent. Although not directly comparable to the results of Priscu et al. (29) due to differences in the position of 16S rDNA nucleotides sequenced, bacterial isolates and 16S rDNA sequences originating from the α- and β-proteobacteria and actinobacteria were similarly detected in both studies. It also seems noteworthy that sequence

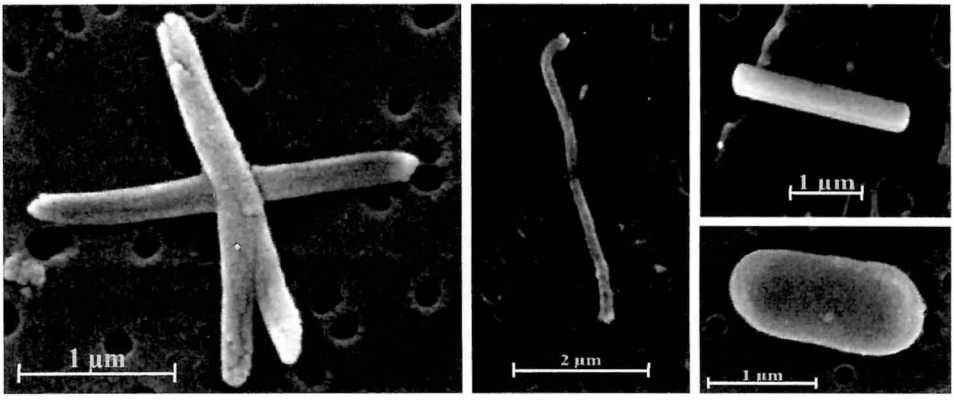

FIGURE 15.5. Scanning electron micrographs of materials filtered from meltwater from Lake Vostok deep ice core section 3593. The particulates shown, apparently bacteria, are retained on the surface of a 0.2 µm isopore (millipore) filter.

pA419 originated from an α-proteobacterium whose nearest 16S rRNA neighbor is an isolate recovered 400 mbs of Lake Baikal (Russia) (4). Only very tenuous extrapolations can be made from 16S rDNA sequences, but the results obtained suggest that Lake Vostok is seeded with bacteria related to those surviving for extended periods in glacial ice, and is probably inhabited by species similar to those found in other permanently cold environments.

Conclusions

Microorganisms recovered from glacial ice are likely to have already endured desiccation, solar irradiation, freezing, a period of frozen dormancy, and thawing. It is not surprising, therefore, that many of the ice core isolates are pigmented and belong to bacterial groups that differentiate into spores that specifically confer resistance to such environmental abuse and facilitate long-term survival under nongrowth conditions. Many also have thick cell walls and polysaccharide capsules and have been demonstrated to be more resistant to repeated cycles of freezing and thawing than standard laboratory bacterial species. Interestingly, closely related bacteria have been recovered from glaciers separated by great distances, suggesting the possibility that some species may indeed have evolved features that help their survival and, conceivably, may even facilitate growth under freezing conditions. Thin films of liquid water may exist between ice crystals, even within apparently solid ice (28), and studies of permafrost (see ref. 31 and chapter 7, this volume), basal glacial ice (34), frozen bacterial suspensions (11), and surface snow (9) have all demonstrated microbial activity under freezing conditions. Evidence for microbial activity

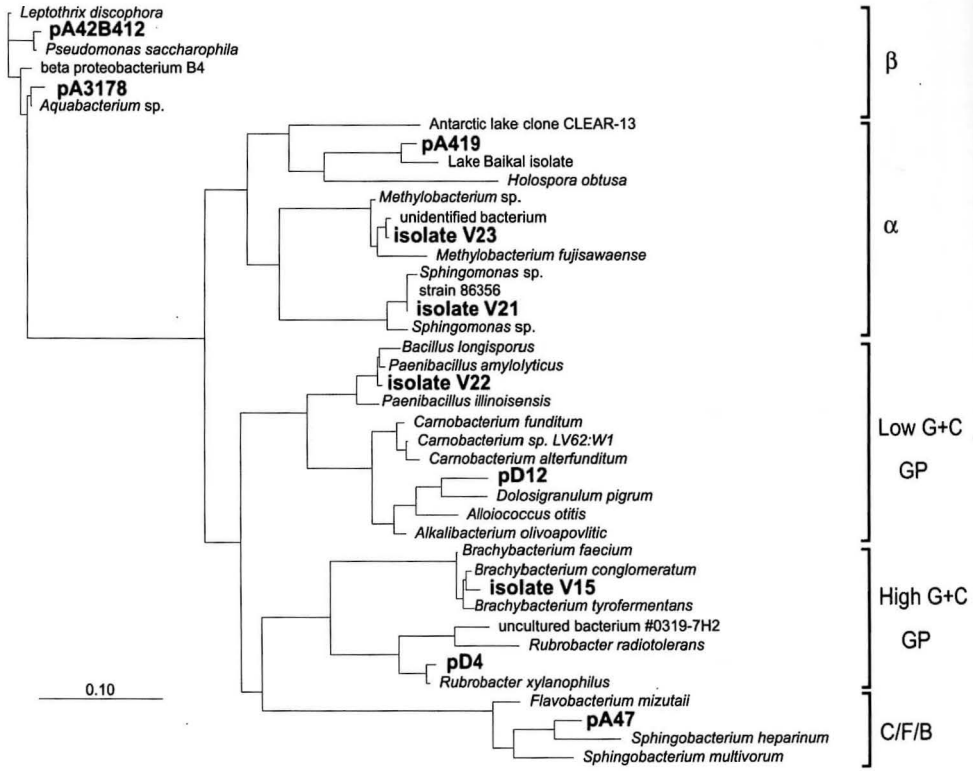

FIGURE 15.6. Phylogenetic analysis of 16S rDNA sequences isolated from bacteria and directly amplified from meltwater from Lake Vostok core section 3593. Sequences that correspond to nucleotides 515 through 1392 of the *E. coli* 16S rDNA were obtained, aligned, and used to construct the figure shown as Figure 4 (25). A best fit tree was created using maximum likelihood with a 771 nucleotide mask of unambiguously aligned positions using fastDNAml (18).

within glacier ice, as implied by geochemical anomalies in polar and nonpolar glacial ice cores (7, 35, 36, 37), provides yet another example of the extreme conditions in which life can exist, extends the known boundary of the biosphere into icy environments, and suggests that *in situ* biological alteration of gases and ions may skew paleoclimatic interpretations of ice core records.

Ice cores from low-latitude, high-altitude glaciers generally contain more recoverable bacteria than polar ice cores, presumably because the Andes and Himalayas are closer to major sources of airborne biological materials. Similarly, polar ice from regions adjacent to the exposed soils and rock surfaces in Taylor Valley (Antarctica) contains more recoverable bacteria than polar ice from remote regions. We have established that bacteria remain viable when

frozen in glacial ice for > 500,000 years and, based on other studies of *Bacillus* spore longevity (8,40), this is almost certainly an underestimate. It is also possible that some microorganisms might even maintain some metabolic activity while apparently frozen within ice.

By identifying and counting the microorganisms present in glacial ice of very different ages, we may be able to relate climate change and geography to local airborne microbial populations (see chapter 6). Similarly, by characterizing individual isolates, we can obtain information that contributes to discussions of the possibility that microorganisms might survive frozen in extraterrestrial environments. These isolates should also provide data that are directly relevant to discussions of the prevalence of antibiotic resistance before the advent of antibiotic therapies, and the survival of life through "Snowball Earth" events (20).

Acknowledgments

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