

Recovery and Identification of Viable Bacteria Immured in Glacial Ice

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An extraction system has been constructed that melts ice from the interior of ice cores and collects the resulting water aseptically. Using this system, bacteria entrapped in ice cores from different geographic locations, that range in age from 5 to 20,000 years old, have been isolated and characterized. Ice cores from the Guliya ice cap on the Tibetan Plateau (China) contained the highest number of colony-forming units per milliliter (~ 180 cfu ml⁻¹) and representatives of many different bacterial species. Much lower numbers of bacteria (> 20 cfu ml⁻¹) were recovered from Sajama (Bolivia) ice cores, although in general such nonpolar ice cores contained more culturable bacteria than samples of polar ice, presumably due to the closer proximity of major biological ecosystems. More bacteria were recovered from Late Holocene ice from the Taylor Dome region than from ice of the same age from the Antarctic peninsula or from Greenland. Bacterial isolates were identified, in terms of their closest phylogenetic relatives, by determining small-subunit ribosomal RNA-encoding DNA sequences (16S rDNAs), and most were related to spore-forming *Bacillus* and *Actinomyces* species, or to nonsporulating Gram positive bacteria. The numbers of recoverable bacteria did not correlate directly with the age of the ice, indicating that most bacteria were deposited episodically in snowflakes and/or attached to larger particles of inorganic and organic debris. By identifying the features that facilitate microbial survival within terrestrial ice, extrapolations to the likelihood of microorganisms surviving frozen in water ice on Mars, Europa, or within comets will be improved. © 2000 Academic Press

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INTRODUCTION

Life is thought to have originated on Earth ~ 4 billion years ago, at a time when Mars had a comparable planetary environment (McKay and Stoker 1989, Brack and Pillinger 1998).

Early in its history, Mars had an atmosphere that generated a substantial greenhouse effect, sufficiently warming the planet to allow the presence of liquid water. As the atmosphere of Mars dissipated due to insufficient planetary mass, the temperature dropped, freezing all water on the surface. However, liquid water may have persisted on the surface of Mars for 10^9 years (Pollack *et al.* 1987), a length of time sufficient for the evolution of microbial life on Earth. If life evolved similarly on Mars before global freezing, then microorganisms may still exist in the subsurface, or possibly closer to the surface in the polar regions. Microorganisms survive on Earth for extended periods frozen in soils (Gilichinsky *et al.* 1993, Shi *et al.* 1997), in perennial lake ice (Olson *et al.* 1998, Paerl and Priscu 1998, Priscu *et al.* 1998, Takacs and Priscu 1998), and in Antarctic rock (Nienow and Friedmann 1993), but only a few studies have focused on microorganisms trapped directly in glacial ice (Abyzov *et al.* 1982, Abyzov 1993, Dancer *et al.* 1997). No attempts have been made to relate these microorganisms to the local geography or climate conditions during deposition, nor have such isolates been systematically investigated for features consistent with increased survival during freezing and persistence while frozen. To address these issues, we are investigating bacteria in ice cores retrieved and archived by the paleoclimate reconstruction program at the Byrd Polar Research Center (BPRC) at Ohio State University. The BPRC collects ice cores of different ages, from both poles and from high-altitude, low-latitude glaciers to establish past global and regional climate changes. These ice cores are, however, also available as a resource to determine the longevity and composition of microbial populations in ice collected from a variety of different ecological sources, deposited at known times and under defined environmental conditions. Here we report the initial results obtained from ice cores that ranged from 5 to 20,000 years in age

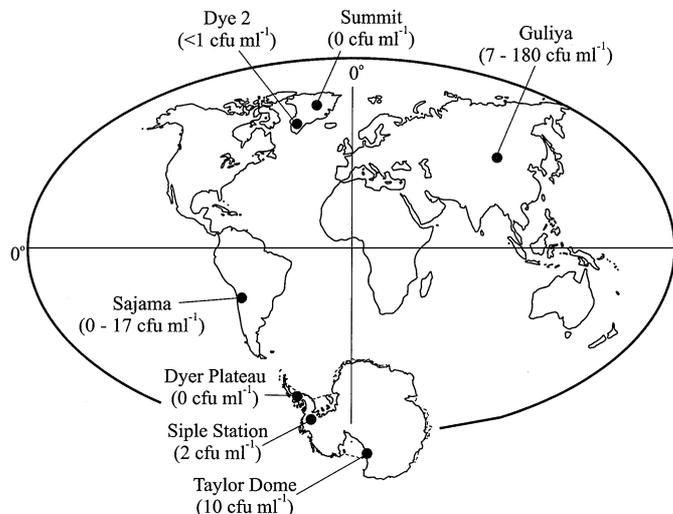


FIG. 1. Location of sampling sites with the number of colony forming units (cfu) per milliliter isolated from ice cores from that region indicated in parentheses.

that were collected from Greenland, China, Peru, and Antarctica (Fig. 1).

RESULTS

Sampling Technology and Controls for Contamination

Ice core exteriors are likely to be contaminated during drilling and subsequent handling, and therefore an autoclavable sampling system was constructed to melt and collect water only from the interior of cores (Fig. 2). Cross-sectional cuts, made with a dust-free bandsaw used exclusively for this purpose, removed a few millimeters of ice from the end of an ice core exposing previously unhandled ice and providing an uniform, flat surface for disinfection by 2 minutes exposure to 95% ethanol. Disinfection by exposure to ultraviolet (UV) light irradiation and to sodium hypochlorite was also considered. However, ethanol treatment had the advantages that the ethanol remained liquid at -20°C , ice cores did not fracture even when soaked for longer times in ethanol to dissolve away potentially contaminated ice, and the ethanol was easily diluted subsequently to nontoxic concentrations. In reconstruction experiments, *Serratia marcescens* cells were swabbed onto the cut surface and onto the saw blade used to cut the ice core, but *S. marcescens* was not then isolated from the core, after the ethanol treatment, by the culture procedures used in this study. Based on these results, the ethanol treatment was effective in killing *S. marcescens* cells on the ice core surface but this treatment would not be expected to kill bacterial endospores or to destroy nucleic acids. Therefore, to monitor the sampling procedure, the cut surface of each ice core was swabbed after the ethanol treatment and before melting, and these swabs were used to inoculate cultures. To date, on only one occasion has a culture been generated from an ethanol treated ice-core surface, and this was identified as a close relative of *Bacillus subtilis*, a

known endospore-forming species. To evaluate the amount of DNA present after ethanol treatments, the cut surfaces of four ice cores were swabbed, and the material collected on the swabs was resuspended by vigorous shaking in 1 ml of sterile water. DNA was extracted (More *et al.* 1994) from the resuspended material and amplified by the polymerase chain reaction (PCR) using universal 16S rDNA primers (see below). This amplification failed to generate PCR products, consistent with the amount of DNA remaining on the cut, ethanol-treated ice-core surfaces being below that detectable by standard PCR procedures.

Recovery, Growth, and Identification

To minimize the possibility for contamination, all post-sampling manipulations were undertaken within an UV-sterilized laminar flow hood, pipettes and work surfaces were cleaned using a sodium hypochlorite (2400 ppm) solution, and all materials were autoclaved twice. Melt water (100–250 ml) was filtered and the $0.2\ \mu\text{m}$ pore-size filter was then vortexed in 5 ml of phosphate buffered saline to resuspend filter-trapped cells. Aliquots of this suspension were used to inoculate a variety of different agar-solidified media held in Petri dishes that were inverted and incubated aerobically at 10 and 25°C for extended periods of time. Higher numbers of colonies were routinely observed on media containing low levels of nutrients, such as R2A, *Actinomycetes* isolation agar, and nutrient agar diluted 100-fold below the manufacturers' recommended concentrations, than on nutrient-rich tryptic soy or tryptose blood agar plates (Difco, USA). Apparently, aged cells need time to synthesize enzymes to repair cell damage accumulated during long periods of exposure to environmental irradiation and to detoxify metabolic byproducts, such as hydrogen peroxide, superoxide, and free radicals (Dodd *et al.* 1997) before they can grow. Colonies often appeared only after 30 to 60 days of incubation at 25°C , or after >100 days of incubation at 10°C , although many of the isolates that formed these colonies then subsequently formed colonies within 2 to 3 days when subcultured on the same growth medium at 25°C . It seems possible that when plated on low nutrient media, damaged cells have sufficient time for cell repair before initiating growth, whereas cells plated on rich nutrient media attempt to grow before they have effectively repaired all the accumulated cell damage.

Bacterial isolates were categorized in terms of colony morphology, growth temperature optima, ability to grow on different media, and provisionally identified by determining 16S rDNA sequences that corresponded to regions between nucleotides 515 and 1492 of the *Escherichia coli* 16S rDNA sequence. Many of the isolates formed highly pigmented colonies, consistent with the presence of pigments providing protection from solar irradiation during atmospheric transport and on the surface of a glacier. Interestingly, some isolates with identical 16S rDNA sequences formed colonies with different morphologies and pigments.

Based on having a 16S rDNA sequence $>95\%$ identical to that of an established species, glacial ice isolates have been assigned to the bacterial genera *Arthrobacter*, *Aureobacterium*, *Bacillus*,

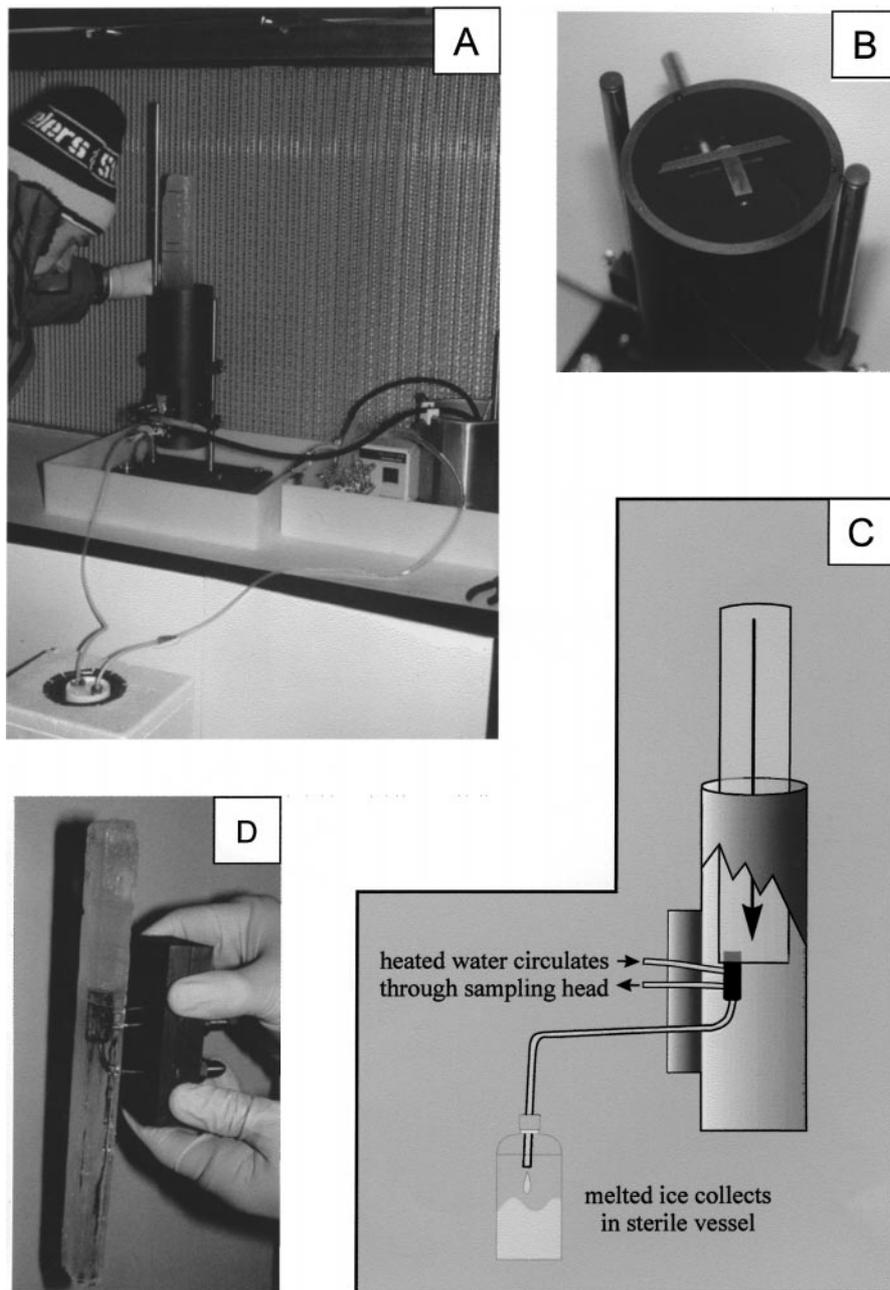


FIG. 2. Ice-core sampler. (A) The complete unit with an ice core inserted perpendicularly into the ice-melting unit. All components of the system are sterilized by autoclaving and then assembled inside a laminar flow hood housed in a -20°C walk-in freezer. (B) Moveable separation flanges facilitate melting half or quarter core sections and allow duplicate samples to be collected from parallel regions through the same core. (C) An ethanol-treated cut core surface is placed in contact with the funnel-shaped sampling head which is heated by circulating water and melted upward through the ice. The water generated is collected directly through a hole in the center of the melting head and pumped into an external sterile container. (D) The sampling head after movement through a core and removal of a cylindrical section of the core interior.

Bradyrhizobium, *Brevibacterium*, *Cellulomonas*, *Clavibacter*, *Flavobacterium*, *Frankia*, *Friedmanniella*, *Methylobacterium*, *Microbacterium*, *Micrococcus*, *Micromonospora*, *Mycobacterium*, *Nocardia*, *Nocardioidea*, *Paenibacillus*, *Planococcus*, *Propioniferax*, *Sphingomonas*, *Staphylococcus*, and *Stenotrophomonas* (Table I). The majority have close phylogenetic re-

lationships to either endospore-forming *Bacilli*, spore-forming *Actinomycetes*, or nonsporulating Gram positive species. Seven of the nine isolates investigated from Antarctic ice cores have 16S rDNA sequences that are less than 90% identical to those of known *Acinetobacter*, *Bacillus*, *Bosea*, *Nocardioidea*, and *Sphingomonas* species, whereas 5- and 200-year-old Guliya ice

TABLE I
Bacterial Isolates from Glacial Ice Cores

Isolate ^a	Core origin and age ^b	Isolate ^a	Core origin and age ^b
<i>Acinetobacter calcoaceticus</i>	TD1.8K ^c	<i>Frankia</i> sp. str. AVN175	SB100
<i>Acinetobacter radioresistens</i>	G5, SB150 ^c	<i>Friedmanniella antarctica</i>	SB12K
<i>Arthrobacter agilis</i>	G5, G200	<i>Methylobacterium</i> sp. str. GK101	TD1.8K
<i>Arthrobacter bakeri</i>	G5	<i>Microbacterium aurum</i>	G200
<i>Arthrobacter globiformis</i>	G200 ^c	<i>Microbacterium lacticum</i>	SB12K
<i>Aureobacterium liquefaciens</i>	G5	<i>Micrococcus lylae</i>	SB20K
<i>Aureobacterium testaceum</i>	SB12K	<i>Micromonospora purpurea</i>	G200
<i>Bacillus firmus</i>	G200	<i>Mycobacteria komossnese</i>	SB12K
<i>Bacillus globisporus</i>	SB100	<i>Nocardia corynebacteroides</i>	SB12K
<i>Bacillus licheniformis</i>	SB100	<i>Norcardioides jensenii</i>	SP150
<i>Bacillus pseudomegaterium</i>	SB100 ^c	<i>Norcardioides plantarum</i>	SB12K
<i>Bacillus sporothermodurans</i>	G200	<i>Paenibacillus amylolyticus</i>	G5
<i>Bacillus subtilis</i>	G5, G200, SP150 ^c	<i>Paenibacillus lautus</i>	G200, SB150 ^c
<i>Bacillus thuringiensis</i>	G5, SB100, SB12K	<i>Paenibacillus polymyxa</i>	G5
<i>Bacillus</i> sp.10	G200, SB100, SB150 ^c	<i>Planococcus kocuri</i>	SB150
<i>Bosea thiooxidans</i>	TD1.8K ^c	<i>Propioniferax innocua</i>	G200
<i>Bradyrhizobium japonicum</i>	TD1.8K	<i>Sphingomonas capsulata</i>	TD1.8K ^c
<i>Brevibacterium acetyllicum</i>	SB12K	<i>Sphingomonas paucimobilis</i>	TD1.8K ^c
<i>Cellulomonas hominis</i>	SB12K	<i>Sphingomonas</i> sp. str. A175	SP150
<i>Cellulomonas turbata</i>	G200	<i>Staphylococcus aureus</i>	G5
<i>Clavibacter michiganensis</i>	G5, G200, SB12K ^c	<i>Staphylococcus hominis</i>	SB12K
<i>Flavobacterium okeanokoites</i>	G5	<i>Stenotrophomonas africana</i>	G5

^a The bacterial species listed are the nearest known phylogenetic neighbor to the isolate based on similarities of partial 16S rDNA sequences (Maidak *et al.* 1999), established by using the ShowDistance function of the beta version PAUP 4.0.

^b G designates ice from Guliya (China), SB ice from Sajama (Bolivia), and TD and SP ice from Taylor Dome and Siple (Antarctica). The numbers following the location designation are the age of the ice in years, or 1000s (K) of years.

^c Isolates with <95% rDNA sequence similarity to the phylogenetic neighbor listed.

isolates had 16S rDNA sequences that are almost identical to those of *B. subtilis*, *Arthrobacter agilis*, and *Clavibacter michiganensis*. An isolate closely related to *C. michiganensis* was also obtained from 12,000-year-old Sajama ice samples. Fungi were also cultured from ice samples from all geographical locations, but their identification has not yet been undertaken.

Geographical and Chronological Variation

Ice cores of different ages from Greenland, China, Peru, and Antarctica (Fig. 1) were sampled to survey the abundance and range of different bacterial species that could be isolated as colonies on agar-solidified media. Colonies were not obtained from 150-year-old ice from the Antarctic Peninsula or from 1500-year-old ice from the Sajama ice cap (Bolivia), whereas ~180 cfu ml⁻¹ were recovered from 200-year-old ice from the Guliya ice cap (China). Low but similar numbers of colony-forming units (>20 ml⁻¹) were recovered from both modern and 12,000- to 20,000-year-old ice from Sajama, indicating that the age had little effect on the number of recoverable bacteria in ice from this region. Late Holocene (1800 years old) polar ice from Taylor Dome (Antarctica) similarly contained only ~10 cfu ml⁻¹, but this was nevertheless a number higher than that cultured from ice of the same age from the Antarctic Peninsula or from Greenland (Summit and Dye 2). Similarly, low numbers of isolates (1–5 cfu ml⁻¹) were reported by Dancer *et al.* (1997)

in glacial ice from the Canadian high Arctic after enrichment for coliform bacteria, and even lower numbers (<1 cfu ml⁻¹) were reported in earlier surveys of polar ice (Abyzov *et al.* 1982, Hardfield *et al.* 1992). It is important to note that variations in snowfall and compression with age mean that 1-ml aliquots of water generated by melting different cores, or different regions of the same core, do not necessarily represent the same period of microbial deposition.

Nucleic Acid Quantitation

To measure the numbers of microorganisms present in glacial ice without culturing, DNA released from cells collected by filtration of 750- to 1000-ml aliquots of water from 150-year-old Dyer Plateau ice, 200-year-old Guliya and Dye 2 ice, and 1500- and 12,000-year-old Sajama ice (Fig. 1) was probed by slot blot hybridization. The cells were lysed by hot detergent treatment and physical disruption using a bead-beater, and the nucleic acids released and similarly released from 100-fold serial dilutions of an *E. coli* culture, were denatured, transferred, and fixed onto nylon membranes. Following hybridization using a universal 16S rDNA probe (1510–1492), [γ -³²P]-ATP end-labeled, signals were obtained only from the 200-year-old Guliya sample and from the *E. coli* control samples. Based on the *E. coli* standard, the Guliya ice contained ~10⁴ cells ml⁻¹. Consistent with

the positive hybridization signal, the highest numbers of colonies ($\sim 180 \text{ ml}^{-1}$) were also obtained from the Guliya ice, but this number of isolates apparently was only $\sim 1\%$ of the cells present.

Electron Microscopy of Particulates Filtered from Melted Ice

Scanning electron microscopy of material filtered from $\sim 1 \text{ L}$ of water from Taylor Dome ice revealed the presence of micro-

organisms and some larger particles of putative biological origin (Fig. 3), but very little inorganic debris whereas there were much large quantities of apparently inorganic granules, in addition to pollen grains, diatoms, and bacteria with a range of different morphologies in the material filtered from similar volumes of water from Guliya and Sajama ice. Dust particles were also visible macroscopically in these nonpolar glacial ices, typically in layers apparently representing the annual deposition of particles

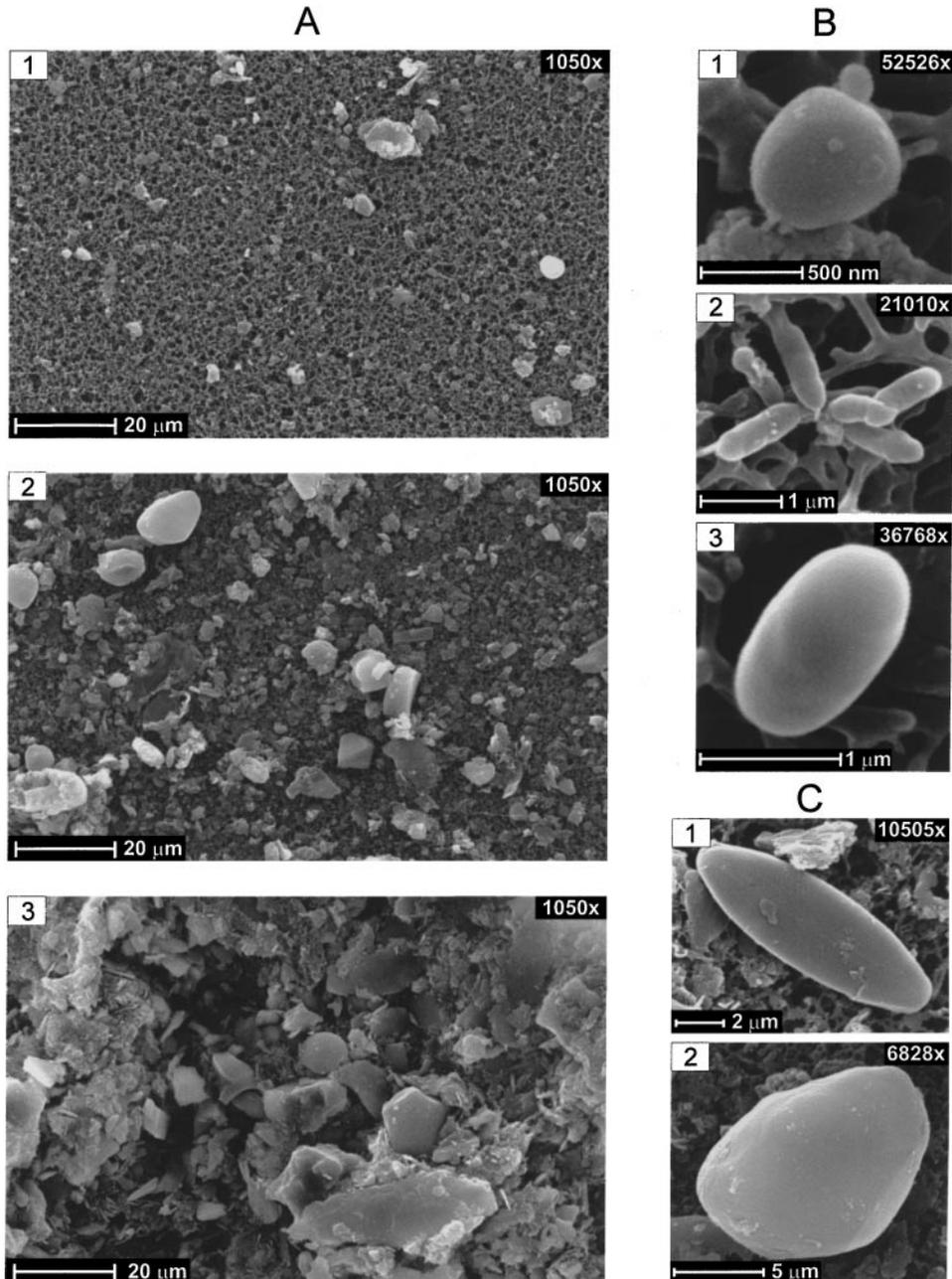


FIG. 3. Microorganisms and particulates filtered from glacial ice cores visualized by scanning electron microscopy. (A) Particles trapped in (1) 1800-year-old ice from Taylor Dome, Antarctica; (2) 12,000-year-old ice from Sajama, Bolivia; and (3) 200-year-old ice from Guliya, China. (B) (1) Coccal, (2) filamentous, and (3) rod-shaped bacteria in 1800-year-old ice from Taylor Dome. (C) (1) Diatom from 12,000-year-old ice from Sajama, and (2) pollen grain from 200-year-old ice from Guliya.

from nearby biological ecosystems. Many of the larger particles trapped in these dust layers would have also transported attached microorganisms into the ice cores, and as insects have also been found frozen in these ice cores, experiments analogous to those that have isolated bacteria from the digestive tracts of insects entombed in amber (Cano and Boruki 1995) should also be possible with ice-core materials.

CONCLUSIONS

Bacteria revived from ice-core samples are likely to have endured desiccation, solar irradiation, freezing, a period of frozen dormancy, and thawing. It is therefore not surprising that a large number of the isolates recovered belong to bacterial groups that form spores, structures known to confer resistance to such environmental abuse. Many also have thick cell walls and polysaccharide capsules that help overcome the stresses associated with water loss, namely increased intracellular solutes, decreased cell size, and a weakened cell membrane, and physical cell rupture caused by freezing and thawing (Fogg 1998). The high frequency of pigment production is also consistent with the need to absorb solar irradiation to prevent lethal DNA damage. Even though the surviving cells may have resistant structures and protective pigments, during extended periods of inactivity, they would still have incurred some radiation and chemical damage. The observation that long periods of incubation were often necessary to obtain colonies is consistent with such aged cells needing time, before beginning growth, to repair this accumulated cell damage.

Ice samples from nonpolar, low-latitude, high-altitude glaciers in the Andes and Himalayas generally contained a larger number of colony-forming units and greater variety of bacterial species than that of polar ice samples, as predicted by their closer proximity to major biological ecosystems. An ice core from Taylor Dome, located at the head of the Taylor Valley in the dry valley complex of Antarctica, was an exception that contained a relatively large number of recoverable bacteria (10 cfu ml^{-1}). Despite the dry, cold conditions, microbiological surveys of this region have documented the abundant presence of cryptoliths, lichen, associations of fungi and bacteria that together inhabit pores in the dominating sandstone (Boyd 1967, Cameron 1971). In this region, with 24 hours of daylight during summer, rock grains are warmed on the surfaces of the frozen lakes and melt into the ice. Pockets of liquid water are created on the surface and within the ice which contain sufficient nutrients to support the growth of microbial communities (Olson *et al.* 1998, Paerl and Priscu 1998, Priscu *et al.* 1998, Takacs and Priscu 1998), many of which are then frozen and trapped completely within the ice during the nonsummer months. This Antarctic dry valley ecosystem, namely microorganisms growing within protective rocks, being transported within rock grains onto ice surfaces and subsequently being entombed and preserved frozen within the ice, seems also a plausible scenario for microbial life on Mars.

The numbers of recoverable bacteria at different positions in ice cores reflect the prevalent climate, wind direction, and individual events that occurred at the time of deposition. There is no consistent, monotonous decrease in the number of recoverable bacteria with increasing age of the ice. For example, Sajama ice deposited $\sim 12,000$ years ago under cool, wet climate conditions (Thompson *et al.* 1998) contained more recoverable bacteria than modern ice, deposited at the same location during a warmer, dryer period. Wet climate conditions increase vegetation density and productivity, increasing the concentration of large airborne particles, such as pollen (Liu *et al.* 1998), that transport microorganisms, and such conditions also decrease desiccation rates. Not surprisingly, the highest numbers of bacteria were always isolated from sections of ice cores that were visibly contaminated with macroscopic debris that presumably transported and protected bacteria. Although most of the bacteria so far isolated are closely related to species found ubiquitously in environmental samples from around the world, some are related to an isolate, strain 34-P, from Antarctic sea ice, or to species of *Bacillus*, *Mycobacteria*, *Micrococcus*, *Brevibacterium*, *Planococcus*, *Arthrobacter*, *Clavibacter*, and *Friedmanniella* that were isolated previously from Siberian permafrost and tundra soil, the Canadian high Arctic, Dry Valley rock and soil, or sea ice (Siebert and Hirsch 1988, Gosink and Staley 1995, Bowman *et al.* 1997, Schumann *et al.* 1997, Shi *et al.* 1997, Zhou *et al.* 1997, Junge *et al.* 1998). The consistent isolation of related microbes from such geographically diverse frozen environments suggests that these species may indeed have features that confer resistance to freezing and extended survival under frozen conditions.

The poles of Mars are covered by ice caps with some physical features similar to those of terrestrial ice sheets (Budd *et al.* 1986, Clifford *et al.* 2000), and although temperatures exceed the frost point and water ice is unstable at lower latitudes, ice may still be present in the nonpolar regions kilometers below the surface (Squyres and Carr 1986). Dust particles from the surface, elevated into the atmosphere by wind, serve as condensation nuclei for carbon dioxide and water which is precipitated, perhaps seasonally, in the polar regions (Clifford *et al.* 2000). The presence of alternating clean and dusty layers within the polar ice could reflect changes in the levels of atmospheric dust, or may result from the sublimation of frozen volatiles during periods of high obliquity. Although organic molecules are likely to be destroyed by the high levels of UV irradiation and peroxides photochemically generated in the martian soil, biochemical traces of life or even viable microorganisms may well be protected from such destruction if deposited within polar perennial ice or far 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) may result in discharges of liquid water that could create environments with ecological niches similar to those inhabited by microorganisms in terrestrial polar and glacial regions. Periodic effluxes of hydrothermal heat might also move microorganisms from below to the martian

surface. 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). Documenting and understanding the survival of terrestrial microorganisms in glacial ice provides an experimentally tractable analog that can be used to evaluate the likelihood of microorganisms surviving frozen in extraterrestrial environments. Characterization of the structures and metabolism of species that are most frequently isolated from ice cores may well provide important clues to lifestyles that might be encountered on Mars, Europa, or within comets.

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