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Brief report

Bacterial recovery from ancient glacial ice

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Summary

Ice that forms the bottom 18 m of a 308 m ice core drilled from the Guliya ice cap on the Qinghan-Tibetan plateau in Western China is over 750 000 years old and is the oldest glacial ice known to date. Fourteen bacterial isolates have been recovered from samples of this ice from ~296 m below the surface (mbs). Based on 16S rDNA sequences, these are members of the α- and β-proteobacterial, actinobacterial and low-G +C Gram-positive bacterial lineages. 16S rDNA molecules have also been amplified directly, cloned and sequenced from the ice-core melt water. These originated from *Pseudomonas* and *Acinetobacter* γ-proteobacterial species. These results demonstrate that bacteria can be recovered from water ice that has frozen for time periods relevant to biological survival through terrestrial ice ages or during interplanetary transport.

Introduction

Glacial ice is formed from compacted snow at the poles, and at many globally distributed low latitude, high-altitude locations. Samples of the atmosphere and particulates prevailing at the time of snowfall are preserved chronologically, and ice core analyses have been used to document and date past climate changes, geological events and human activities (Lorius *et al*., 1985; Dansgaard *et al*., 1993; Grootes *et al*., 1993; Thompson *et al*., 1997; 1998; 2000; Petit *et al*., 1999). Viruses, bacteria, fungi, pollen grains and seeds have been identified in glacial ice (Abyzov, 1993; Dancer *et al*., 1997; Abyzov *et al*., 1998; Castello *et al*., 1999; Willerslev *et al*., 1999; Christner *et al*., 2000; Zhang *et al*., 2001), but few attempts have been made to establish their viability, diversity or longevity. Abyzov (1993) was able to culture several different bacterial species, yeasts and filamentous fungi from sections of a Vostok ice core that were less than 12 000 years old, but recovered only small numbers of exclusively endospore-forming bacteria from ~200 000-year-old sections of this core. The oldest glacial ice recovered to date was obtained in 1992 from ~300 mb of centre of the Guliya ice cap in Tibet (Thompson *et al*., 1997; Fig. 1A). At the time of collection, the bedrock temperature was −2.1°C and the surface temperature ranged from −10°C to −20°C. Without thawing, sections of the ice core were sealed in plastic tubes which were then placed inside aluminum covered cardboard tubes. These were immediately air-freighted under close supervision directly to the Byrd Polar Research Center (BPRC) at The Ohio State University. During the 2 day transit period, the temperature of the ice never exceeded −5°C, and since then sections of this ice core have been kept in the aluminium-covered tubes at −20°C to −30°C in the BRPC ice-core storage facility. Samples of this ice have been subjected to very extensive visual, chemical and physical investigations (Thompson *et al*., 1997) and, based on the absence of lateral distortions or fractures and no residual ^{36}Cl, the ice and the materials trapped within the ice from ~290 mb are at least 750 000 years old. Here we report the recovery and identification of bacteria, and direct amplification of DNA molecules from melt water generated from samples of this ancient ice.

Two sequential 78 cm vertical sections of the ice core from ~296 mb of the Guliya ice cap were consumed for this study. A dust-free band saw was used to cut an outer layer from these sections at −5°C to expose previously unhandled ice. This was then washed with 0.22 μm filtered 95% ethanol at −5°C until a surface layer equivalent to ~10% of the mass of the ice had been removed. After rinsing with twice-autoclaved, deionized water, the remaining ice was placed in sterile containers and allowed to thaw at 4°C. Within 6 h of melting, particulates were collected from ~280 ml of melt water by filtration using

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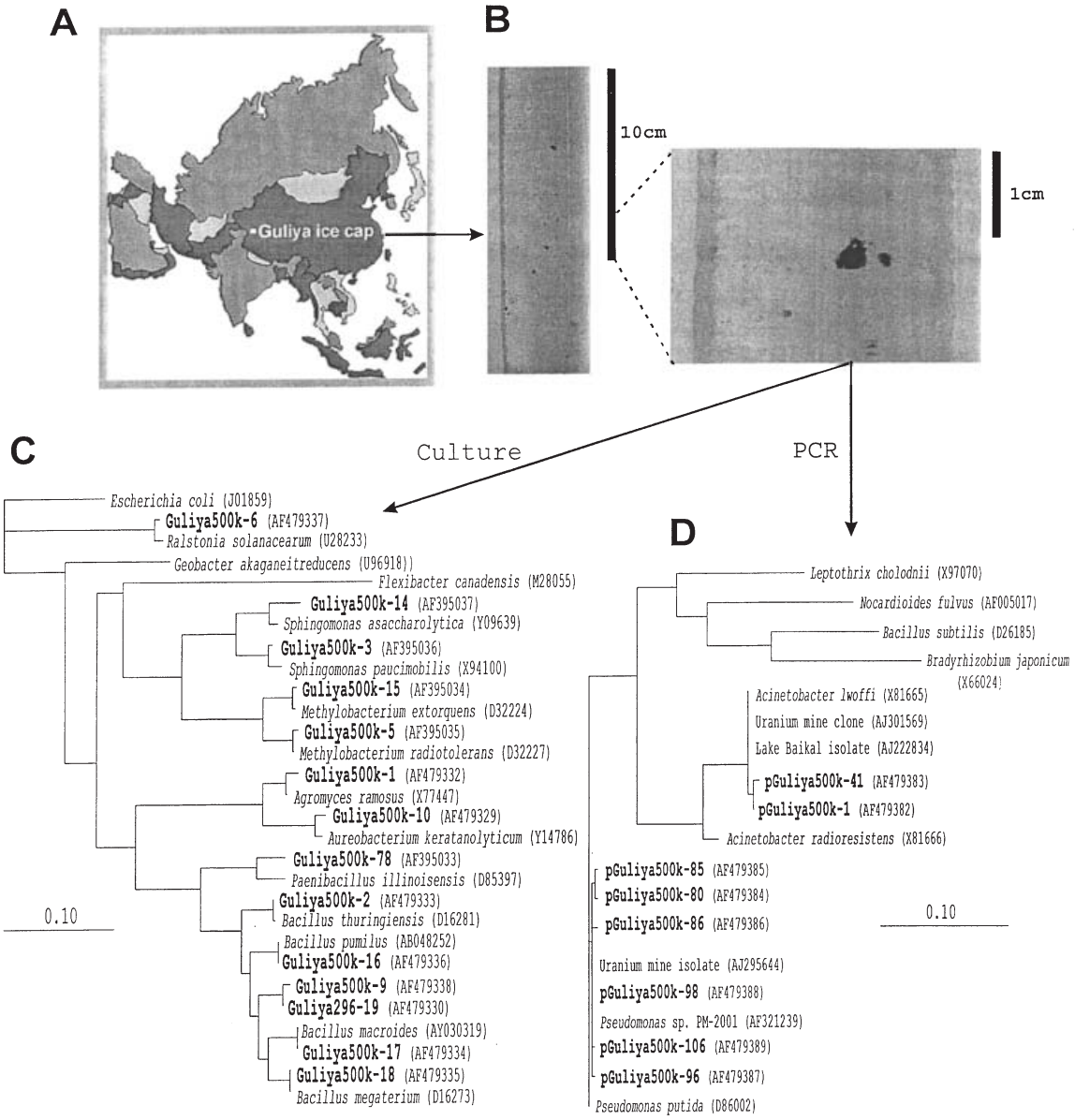

Fig. 1. (A) Drill site and (B) photographs of a section of the ice core used in this study. This ice originated from ~296 mb of the Guliya ice cap (35°17 min N, 81°29 min E; ~6400 m above sea level) on the Qinghai-Tibetan Plateau. Chemical analysis revealed that the macroscopic particles present were inorganic material and given the depletion of chloride, sulphate and nitrate ions in the surrounding ice, they did not originate as part of a contamination event from the underlying bedrock. They were apparently deposited as dust particles on the glacier's surface, trapped by subsequent snowfall and then immured in the resulting glacial ice for >750 000 years (Thompson *et al.*, 1997).

C. The sequences of 16S rDNA amplicons generated from the cells that formed a single colony, corresponding to nucleotides 27 through 1492 of the *E. coli* 16S rDNA, were imported into the ARB software environment (Strunk *et al.*, 1998) and aligned, based on secondary structures, with all sequences available in the ribosomal database project (Maidak *et al.*, 2001) and GenBank (Benson *et al.*, 2000). The maximum likelihood tree shown was generated using fastDNAml (Olsen *et al.*, 1994) with a 1371 nucleotide mask of unambiguously aligned positions. The isolates were designated by their origin (Guliya), age (500 k year) and have individual isolate numbers (1, 2, etc.). The scale bar indicates 0.10 fixed substitutions per nucleotide position. GenBank accession numbers are given in parentheses.

D. Amplicons, corresponding to nucleotides 515 through 1392 of the *E. coli* 16S rDNA were generated from concentrated filtrate, cloned and sequenced. Eight sequences were obtained, in most cases several times, and designated pGuliya500k-x with p indicating a cloned DNA rather than an isolate and x the clone number. The maximum likelihood tree shown was generated as in (C) but using a 426 nucleotide mask of unambiguously aligned positions. The scale bar indicates 0.10 fixed substitutions per nucleotide position. The cloned sequences were most similar, exhibiting from 99.2% to 100% identity, to sequences and isolates from other subsurface environments. Specifically, six of the cloned sequences were 98.9% to 99.4% identical to the 16S rDNA sequence of *Pseudomonas putida* (D86002), and two were 96.3% and 99.2% identical to the 16S rDNA sequence of *Acinetobacter lwoffi*.

0.22 μm Isopore filters (Millipore). The particulates were re-suspended by gentle agitation of the filters in 5 ml of phosphate-buffered saline, and aliquots of the resulting suspensions were used to inoculate enrichment cultures, were spread on agar-solidified media and were used for DNA extraction for PCR amplification. The 0.22 μm filtrates were also collected, concentrated by centrifugation through Biomax-100 centricon plus-80 units (Millipore), and aliquots of the resulting concentrated solutions were also used as templates in PCR amplifications.

Liquid enrichment cultures, and agar-solidified media in plates were inoculated with aliquots of the resuspended ice core particulates. Duplicates of all cultures and plates were incubated at 4°C and 22°C for at least 100 days, and in some cases for >1 year. Under aerobic incubation conditions, tryptose blood, actinomycetes isolation, 100% and 1% nutrient broth, 100% and 1% tryptic soy broth (Difco, Detroit, MI), R2 (Reasoner and Geldreich, 1985) and M9 (Sambrook *et al*., 1989) glucose-minimal salts media were used, and under anaerobic incubations conditions, media designed to grow methanogens, acetogens and sulphate reducers were used (Christner *et al*., 2000). No growth was observed under anaerobic conditions, and no colonies grew on the directly inoculated plates under aerobic conditions. However, 14 different bacterial isolates were obtained that grew as colonies on plates inoculated with samples taken from the 100% and 1% nutrient broth and 1% tryptic soy enrichment cultures after >30 day aerobic incubation at 4°C. Only one such isolate was obtained from an enrichment culture incubated aerobically at 22°C, but all of the isolates grew subsequently and formed colonies within 5 days on plates incubated at 22°C. The 16S rDNA sequences of these isolates, equivalent to positions 27 through 1492 of the *Escherichia coli* 16S rDNA sequence were determined, revealing that seven were members of the *Bacillus* and *Paenibacillus* endospore-forming lineages, four were α-proteobacteria, two were actinobacteria, and one was a β-proteobacterium (Fig. 1C). They are similar, but not identical to isolates we recovered previously from younger ice cores from Tibet, Bolivia and Antarctica (Christner *et al*., 2000; 2001). It appears therefore that species in these lineages have features, in some cases clearly endospores, that facilitate their survival in frozen environments.

Populations of small subunit rDNA molecules were generated directly from samples of the concentrated melt water filtrates by PCR amplification using combinations of *Bacteria*-specific and universal primers (27F or 515F with 1392R or 1525R), but not when combinations of *Archaea*-specific and universal primers were used (Lane, 1991; Reysenbach and Pace, 1995). Individual molecules were cloned and sequenced from these populations, corresponding to the sequence from nucleotides 515 through 1392 of the *E. coli* 16S rDNA. Eight different sequences were obtained, six that originated from *Pseudomonas* and two from *Acinetobacter* species. Intriguingly, in every case, the most similar 16S rDNA sequence previously documented was from a clone or an isolate from a subsurface environment.

Conclusions

These results document the recovery of viable bacteria from glacial ice that is >750 000 years old. Based on the reports of bacterial survival and recovery from much older samples of amber, salt crystals and permafrost (Cano and Borucki, 1995; Shi *et al*., 1997; Vreeland *et al*., 2000), this is presumably an underestimate of the maximum longevity of bacteria in ice. Nevertheless, these results provide experimentally determined data for discussions of microbial longevity in very cold, frozen environments and for the possible survival of life through the proposed snowball Earth events (Hoffman *et al*., 1998; Kirschvink *et al*., 2000).

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References

Abyzov, S.S. (1993) Microorganisms in the Antarctic ice. In *Antarctic Microbiology*. E.I. Friedmann (ed.), New York: Wiley-Liss, pp. 265–295.

Abyzov, S.S., Mitskevich, I.N., and Poglazova, M.N. (1998) Microflora of the deep glacier horizons of central Antarctica. *Microbiology (Russia)* **67:** 66–73.

Benson, D.A., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J., Rapp, B.A., and Wheeler, D.L. (2000) GenBank. *Nucleic Acids Res* **28:** 15–18.

Cano, R.J., and Boruki, M.K. (1995) Revival and identification of bacterial spores in 25- to 40-million-year old Dominican amber. *Science* **268:** 1060–1064.

Castello, J.D., Rogers, S.O., Starmer, W.T., Catranis, C.M., Ma, L., Bachand, G.D., *et al.* (1999) Detection of tomato mosaic tobamovirus RNA in ancient glacial ice. *Polar Biol* **22:** 207–212.

Christner, B.C., Mosley-Thompson, E., Thompson, L.G., Zagorodnov, V., Sandman, K., and Reeve, J.N. (2000) Recovery and identification of viable bacteria immured in glacial ice. *Icarus* **144:** 479–485.

Christner, B.C., Mosley-Thompson, E., Thompson, L.G., and Reeve, J.N. (2001) Isolation of bacteria and 16S rDNAs from Lake Vostok accretion ice. *Environ Microbiol* **3:** 570–577.

Dancer, S.J., Shears, P., and Platt, D.J. (1997) Isolation and characterization of coliforms from glacial ice and water in Canada's High Arctic. *J Appl Microbiol* **82:** 597–609.

Dansgaard, W., Johnsen, S.J., Clausen, H.B., Dahl-Jensen, D., Gundestrup, N.S., Hammer, C.U., *et al.* (1993) Evidence for general instability of past climate from a 250-kyr ice-core record. *Nature* **364:** 218–220.

Grootes, P.M., Stuiver, M., White, J.W.C., Johnsen, S., and Jouzel, J. (1993) Comparison of oxygen isotope records from the GISP2 and GRIP Greenland ice cores. *Nature* **366:** 552–554.

Hoffman, P.F., Kaufman, A.J., Halverson, G.P., and Schrag, D.P. (1998) A neoproterozoic snowball Earth. *Science* **281:** 1342–1346.

Kirschvink, J.L., Gaidos, E.J., Bertani, L.E., Beukes, N.J., Gutzmer, J., Maepa, L.N., and Steinberger, R.E. (2000) Paleoproterozoic snowball Earth: extreme climatic and geochemical global change and its biological consequences. *Proc Natl Acad Sci USA* **97:** 1400–1405.

Lane, D.J. (1991) 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics.* Stakebrandt, E., and Goodfellow, M. (eds). Chichester, New York: Wiley Press, pp. 133.

Lorius, C., Jouzel, J., Ritz, C., Merlivat, L., Barkov, N.E., and Korotkevich, Y.S. (1985) 150,000-year climatic record from Antarctic ice. *Nature* **316:** 591–595.

Maidak, B.L., Cole, J.R., Lilburn, T.G., Parker, C.T., Jr, Saxman, P.R., Farris, R.J., *et al.* (2001) The RDP-II (Ribosomal Database Project). *Nucleic Acids Res* **29:** 173–174.

Olsen, G.J., Natusda, J.H., Hagstrom, R., and Overbeek, R. (1994) fastDNAml: a tool for construction of phylogenetic trees of DNA sequences using maximum likelihood. *Comput Appl Biosci* **108:** 41–48.

Petit, J.R., Jouzel, J., Raynaud, D., Barkov, N.I., Barnola, J.M., Basile, I., *et al.* (1999) Climate and atmospheric history of the past 420,000 years from the Vostok ice core, Antarctica. *Nature* **399:** 429–436.

Reasoner, D.J., and Geldreich, E.E. (1985) A new medium for the enumeration and subculture of bacteria from potable water. *Appl Environ Microbiol* **46:** 1–7.

Reysenbach, A.-L., and Pace, N.R. (1995) Reliable amplification of hyperthermophilic archaeal 16S rRNA genes by the polymerase chain reaction. In *Archaea: a Laboratory Manual*. Robb, F.T., and Place, A.R. (eds). Cold Spring Harbor Laboratory, New York: Cold Spring Harbor Press.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor Laboratory, New York: Cold Spring Harbor Press.

Shi, T., Reeves, R.H., Gilichinsky, D.A., and Friedmann, E.I. (1997) Characterization of viable bacteria from Siberian permafrost by 16S rDNA sequencing. *Microbiol Ecol* **33:** 169–179.

Strunk, O., Gross, O., Reichel, B., May, M., Hermann, S., Struckmann, N., *et al.* (1998) *ARB: a Software Environment for Sequence Data*. Munich, Germany: Department of Microbiology, Technical University of Munich,

Thompson, L.G., Yao, T., Davis, M.E., Henderson, K.A., Mosley-Thompson, E., Lin, P.N., *et al.* (1997) Tropical climate instability: The last glacial cycle from a Qinghai-Tibetan ice core. *Science* **276:** 1821–1825.

Thompson, L.G., Davis, M.E., Mosley-Thompson, E., Sowers, T.A., Henderson, K.A., Zagorodnov, V.S., *et al.* (1998) A 25,000 year tropical climate history from Bolivian ice cores. *Science* **282:** 1858–1864.

Thompson, L.G., Yao, T., Mosley-Thompson, E., Davis, M.E., Henderson, K.A., and Lin, P.-N. (2000) A high-resolution millennial record of the South Asian Monsoon from Himalayan ice cores. *Science* **289:** 1916–1919.

Vreeland, R.H., Rosenzweig, W.D., and Powers, D.W. (2000) Isolation of a 250 million-year old halotolerant bacterium from a primary salt crystal. *Nature* **407:** 897–900.

Willerslev, E., Hansen, A.J., Christensen, B., Steffensen, J.P., and Arctander, P. (1999) Diversity of Holocene life forms in fossil glacier ice. *Proc Natl Acad Sci USA* **96:** 8017–8021.

Zhang, X., Yao, T., Ma, X., and Wang, N. (2001) Analysis of the characteristics of microorganisms packed in the ice core of Malan Glacier, Tibet. *Science in China (Series D)* **44:** 165–170.