# A late quaternary ice wedge from the Fox Permafrost Tunnel in central Alaska is a time capsule for gas and bacteria

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### Living bacteria were revived in the laboratory from an ice wedge, 24,000 years of age

Ice wedges are among the most common features of permafrost regions in extremely cold, high latitude areas of the world. They grow as the result of repeated cycles of frost cracking followed by the infiltration of snow, meltwater, soil, and other material into the frost cracks. Material incorporated into the ice wedge quickly becomes frozen and the ice, as well as ice in soil and organic particles, can be preserved in a frozen state for thousands of years. Ice wedges are known to be present in the Fox Permafrost Tunnel in central Alaska (Fig. 1). Their shapes and internal fabrics show no signs of thawing (French, 1976), indicating that the ice wedges have been continuously frozen. They might include

microorganisms which have intruded into cracks as suspensions. Microbes already have been found in permafrost (Friedmann, 1994; Gilichinsky et al., 1995) and a viable bacterium has been isolated from ice lenses in Fox Tunnel (Pikta et al., 2005). Although there are a number of cases of bacteria in frozen environments, no investigation has ever been made of the preservation of ancient bacteria within ice wedges, even though these geocryological features are virtually ubiquitous in high latitude areas. The age of ice wedges remained to be established. We now have estimated by radiocarbon dating that the age of this ice wedge in the Fox Tunnel is 24,000 years and have predicted that the isolated bacteria have been entrapped therein for the same period.

## Fox Permafrost Tunnel in central Alaska is an artificial freezer that preserves ice wedges at -4° C

The Fox Permafrost Tunnel in central Alaska near Fairbanks (Johansen et al., 1988) (located N64°57.084' W147°37.250') is a scientific preserve maintained by the Cold Regions Research Laboratory of the U.S. Army. Here, buried, fossilized Pleistocene ice wedges are exposed in the tunnel wall (Fig. 1b) within a complex sequence of loess, paleosoils and ice bodies. The Fox Permafrost Tunnel is kept at a temperature of about -4° C to preserve the permafrost features. More than a dozen large ice wedges, as much as 3-4 m across, occur within the frozen loess exposed within the permafrost tunnel. The large, inactive ice wedges exhibit numerous thin, vertical bands of sediment and ice veinlets (Fig. 1c) characteristic of undisturbed ice wedges, as well as numerous tiny air bubbles (Fig. 1d). The shape and internal fabrics of the ice wedges show no signs of thawing or secondary infilling (French, 1976), indicating that the ice wedges have been continuously frozen since they formed. We collected samples of ice from a large ice wedge in Fox Permafrost Tunnel to determine if microorganisms might have been trapped, frozen and preserved in the ice wedge as it formed.

## Estimated age of the ice wedge is about 24,000 years by radiocarbon dating

We separated and sampled gas preserved within bubbles in the ice wedges. Approximately 2.5 kg of ice from the ice wedge was submerged into a warm solution saturated with sodium chloride and, as the ice melted, 0.7 ml of gas was released from the bubbles and collected. The concentration of methane in the ice wedge gas was 0.8±0.006%, a value several orders of magnitude higher than atmospheric methane concentrations (Morizumi et al., 1996) We are confident that our gas sample within the ice wedge was separated from the atmosphere for thousands of years since we determined that the concentration of the stable isotope $^{13}C$ was -84.651 ‰, thus demonstrating that any contamination by atmospheric air was negligible. A radiocarbon date of 24,884±139 yr BP (data number: NUTA2-3477) was determined for the methane by the Nagoya University Tandetron accelerator mass spectrometry system.

## Over 200 isolates of non-spore-forming bacteria trapped in an ice wedge more than 24,000 years ago were revived in the laboratory

About 40 $cm^3$ of the ice wedge was surface-sterilized by dipping it into a 70% ethanol solution, melted and spread on various kinds of agar media after diluting aseptically. The cultivation media were Luria Broth (LB), LBG (LB medium plus 10 g / l of glucose), R2B (Miteva et al., 2004) (2.0 g / l of yeast extract, peptone, casamino acid, glucose and soluble starch, 1.2 g / l of sodium pyruvate and $K_2HPO_4$, 0.2 g / l of $MgSO_4 \cdot 7H_2O$), a 100-fold diluted LB and LBG, minimal medium (MM) (Stanier et al., 1986) (1 g / l of $K_2HPO_4$ and $NH_4Cl$, 0.2 g / l of $MgSO_4 \cdot 7H_2O$, 0.01 g / l of $FeSO_4 \cdot 7H_2O$, 0.01 g / l of $CaCl_2$, 0.1 mg / l of trace elements), MMG (MM medium plus 5.0 g / l of glucose) and MME (MME-1 and MME-2, containing 0.2 ml / l and 2.0 ml / l of filter-sterilized ice extract obtained from the supernatant of melted ice wedge, respectively). All media contained 20 g/l of agar. Plates were incubated aerobically at 15° C in the dark for 3 months with sealing. We isolated living bacteria at concentrations up to $10^5$ to $10^6$ colony-forming units (CFU) per ml of ice using standard culturing techniques. In total, 248 aerobic or facultatively an-

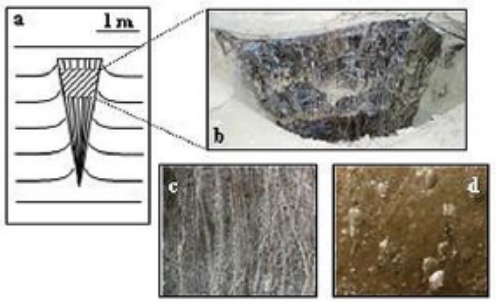
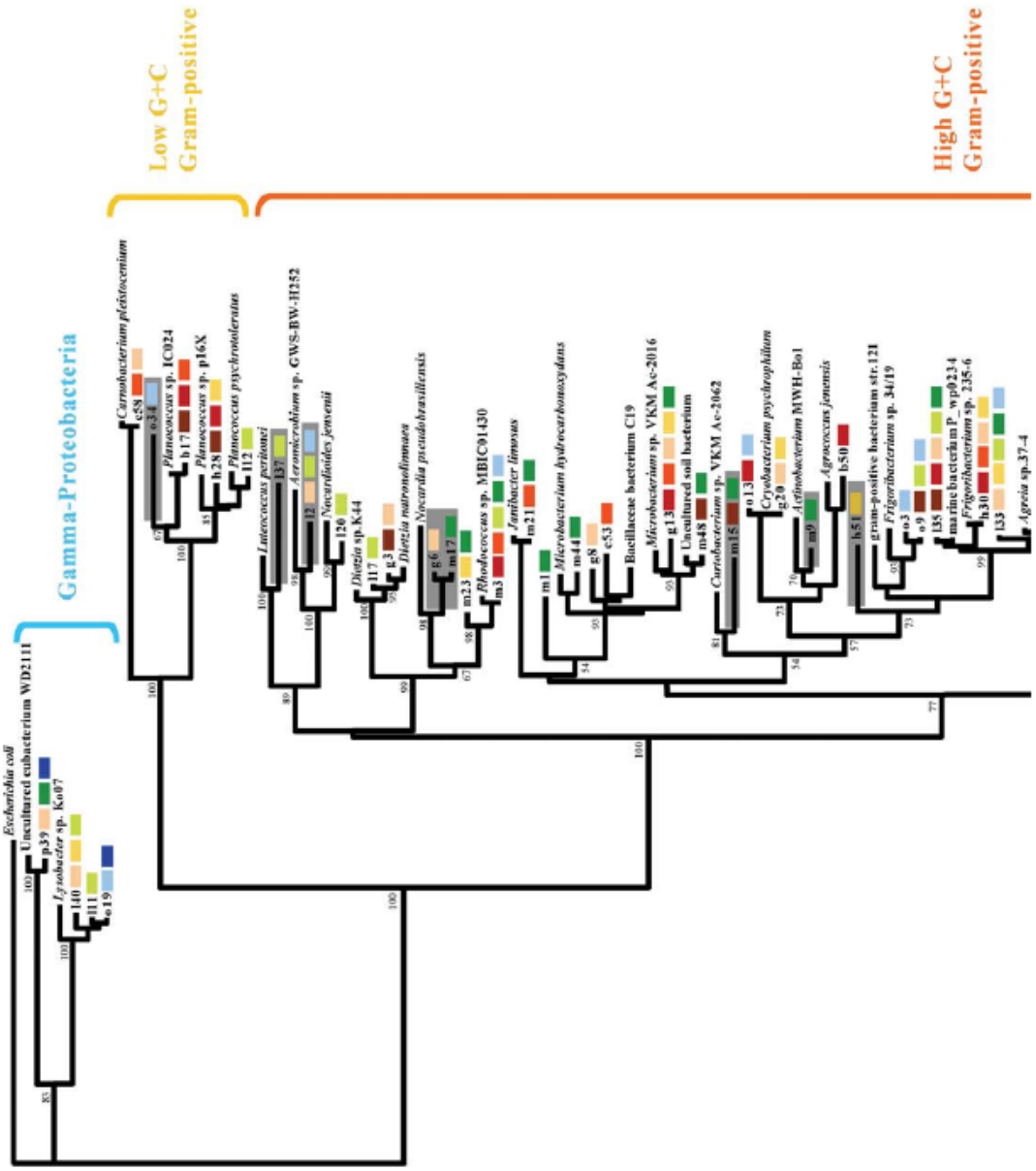
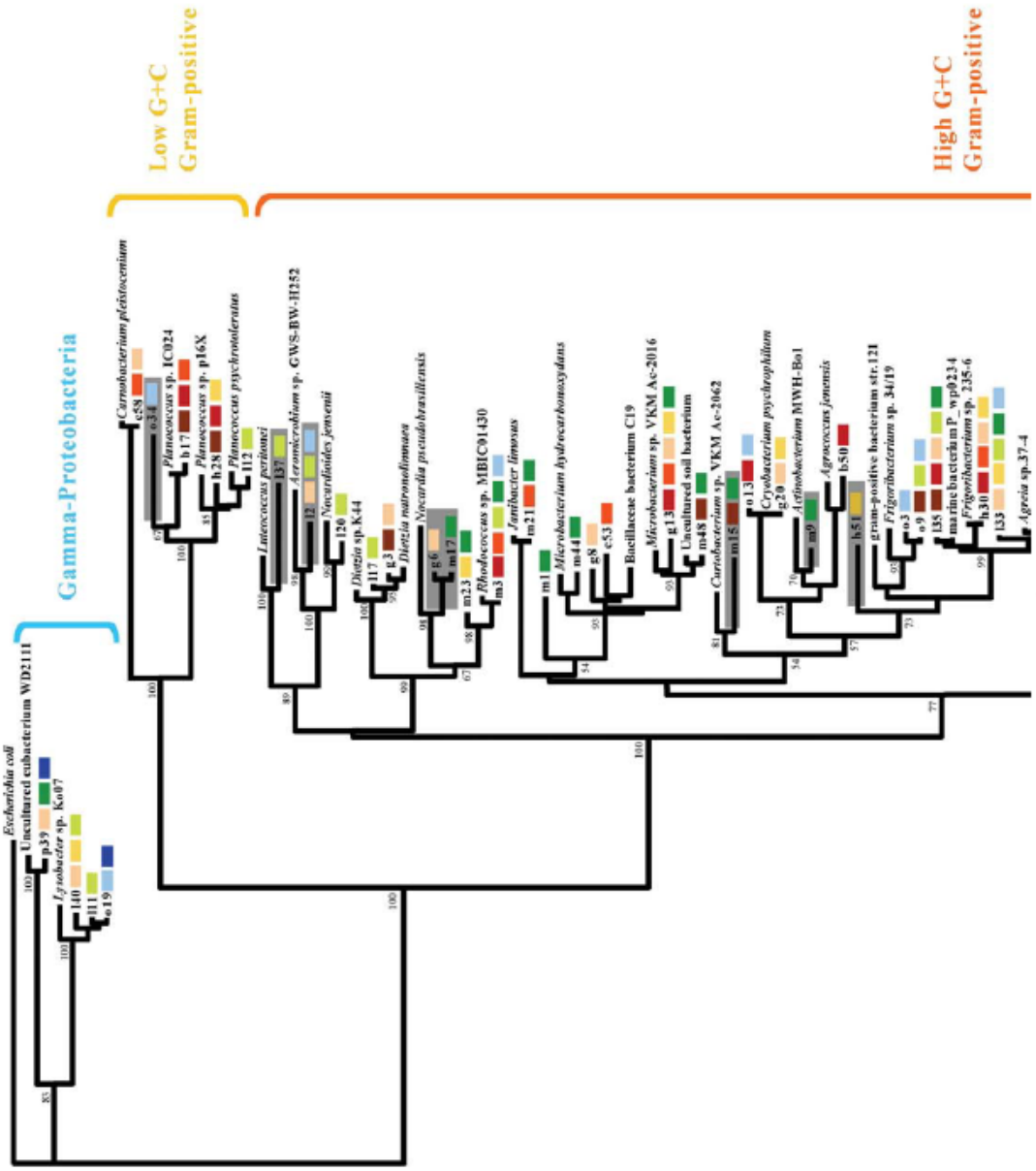



**Figure 1. Fabrics of the ice wedge in Fox Tunnel**
**a, Schematic pattern of ice wedge in permafrost.**
**b, Exposed part of ice wedge.**
**c, Foliation of ice indicating annual veinlets.**
**d, Air bubbles in the ice (1-2 mm in diameter).**





aerobic bacteria were isolated, and were grouped into 62 representative strains by identical sequences of partial 16S rDNA (*Escherichia coli* position 27 to 520). The homological sequences were searched in GenBank by BLAST search (http:/www.ncbi.nlm.nih.gov/). The phylogenetic tree was constructed with 62 representatives and 43 of their closest relatives. They were affiliated with three phylogenetic groups (Fig. 2): high-GC Gram-positive bacteria (79.5%), low-GC Gram-positive bacteria (4.4%) and gamma-Proteobacteria (16.1%). Isolates falling into the high GC group formed the largest cluster in terms of diversity and abundance. These bacteria were affiliated with *Actinomycetales* and belonged to the genera *Arthrobacter* (30.1%), *Brachybacterium* (5.2%), *Rhodococcus*, *Frigoribacterium*, *Microbacterium*, *Brevibacterium* (ea. 4.4%), *Nocardioides* and some others. Bacteria of the low-GC group belonged to the genera *Planococcus* (3.2%) and *Carnobacterium* (1.2%). Eleven representative strains marked as grey boxes in Fig. 2 showed less than 97% homology with partial 16S rDNA. Strains no. l37 and h51 were in a different cluster than their known relatives (*Luteococcus peritonei* (94.8%) and *Agreia* sp.5-1 (95.1%), respectively) and they are possibly novel species.

## A perspective for growth in ice wedge

Twenty nine representative strains isolated from LB, LBG and R2B media were examined for growth at temperatures of -5, 4, 15, 27 and 37° C. All of them grew at 4° C and 15° C and 25 of them were able to grow at 27° C. None grew at 37° C. They are therefore psychrotolerant or psychrophilic according to the definition of Morita (1975). We found that the 10 psychrotolerant representatives grew on agar plates at -5 °C after 2 months. This finding demonstrates that some bacteria isolated from ancient ice had enzymatic activities allowing proliferation at subzero temperature. The dominant group among these isolates was *Actinomycetales*, containing general inhabitants of soils. We suggest that these microbes were carried via soil particles into the ice wedge at the time of formation. This is consistent with the fact that all the isolates were non-spore-forming bacteria, which have been reported to be abundant in permafrost soils (Kochkina et al., 2001; Vorobyova et al., 1997). An intriguing question that remains unanswered is whether the bacterial isolates which we found were in the active or dormant state during the thousands of years they spent sealed in the ice wedge. However, our growth experiments clearly demonstrate a capability for bacterial proliferation in ice wedges, and in frozen permafrost in general. The ice wedges certainly represent some extreme environments for microbes that provide us unique opportunities to study their properties to survive and grow under such conditions and also their capabilities of interest for a wide variety of applications that had not previously been considered.

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