Correspondence

Long-term persistence of bacterial DNA

Eske Willerslev[1,2], Anders J. Hansen[1*], Regin Rønn[1], Tina B. Brand[1], Ian Barnes[2], Carsten Wiuf[3], David Gilichinsky[4], David Mitchell[5] and Alan Cooper[2]

The persistence of bacterial DNA over geological timespans remains a contentious issue. In direct contrast to *in vitro* based predictions, bacterial DNA and even culturable cells have been reported from various ancient specimens many million years (Ma) old [1–8]. As both ancient DNA studies and the revival of microorganisms are known to be susceptible to contamination [8–10], it is concerning that these results have not been independently replicated to confirm their authenticity. Furthermore, they show no obvious relationship between sample age, and either bacterial composition or DNA persistence, although bacteria are known to differ markedly in hardiness and resistance to DNA degradation [11]. We present the first study of DNA durability and degradation of a broad variety of bacteria preserved under optimal frozen conditions, using rigorous ancient DNA methods [8–10]. The results demonstrate that non-spore-forming gram-positive (GP) Actinobacteria are by far the most durable, out-surviving endospore-formers such as Bacillaceae and Clostridiaceae. The observed DNA degradation rates are close to theoretical calculations [9], indicating a limit of ca. 400 thousand years (kyr) beyond which PCR amplifications are prevented by the formation of DNA interstrand crosslinks (ICLs).

The twelve permafrost samples (0-8.1 Ma) investigated were obtained from northeast Siberia and Beacon Valley, Antarctica. DNA preservation at these sites is exceptional due to constant subzero temperatures, largely neutral pH, and anaerobic conditions.

Epifluorescence microscopy revealed ~10^7cells/gram wet-weight in the bacterial size range. The cell counts are in agreement with previous results obtained on permafrost [2,3]. 16S rDNA sequences of 120 bp and 600 bp could be reproducibly amplified from samples up to 400–600 kyr, and show an inverse relationship between PCR amplification efficiency and fragment length that is typical of ancient DNA [8–10,12]. Controls for surface contamination during sampling were negative. Chimeric sequences were excluded from analysis, along with sequences that failed a bootstrap test for independent reproducibility [13]. DNA concentrations and taxonomic diversity were found to decrease with age until 400–600 kyr, at which point the percentage of templates with ICLs reached 100% (Figure 1A–C). Sequences from the older samples appear to be a subset of those from younger material, and all identified bacterial taxa are known soil inhabitants, indicating that permafrost is a non-extremophile environment. There were clear age-related patterns in taxon survival across geographically widespread samples (separated up to 1400 km). Sequences of non-spore-forming GP Actinobacteria, affiliated largely to the genus *Arthrobacter* (99–100% similarity), consistently persisted for the longest time, followed by GP endospore-forming Bacillaceae and Clostridiaceae and finally gram-negative (GN) bacteria, mostly Proteobacteria (Figure 1D).

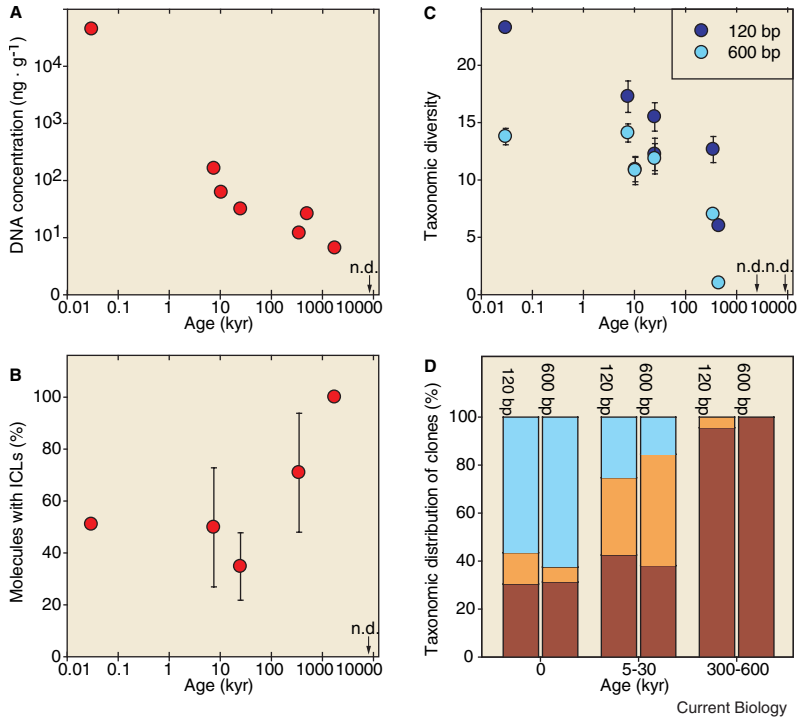

Figure 1. Long term persistence of bacterial DNA.
(A) Bacterial DNA concentrations determined using a Picogreen fluorescence assay as a function of permafrost age. (B) Percentage of interstrand cross-links (ICLs) as a function of permafrost age (means ± standard deviation (s.d.), n = 4). (C) Diversity of sequence groups as a function of permafrost age. Clones ≥96% identical were assumed to belong to the same taxon to account for miscoding lesions and intraspecies sequence heterogeneity [16]. To standardize the number of clones, sequence diversity was estimated in each sample using 1000 datasets of 25 (120 bp) and 16 (600 bp) randomly chosen clones, as these are the sizes of the smallest individual datasets [13]. (D) Frequency of gram-positive Actinobacteria (brown), low-GC gram-positive bacteria (endospore formers; orange) and gram-negative bacteria (blue) largely Proteobacteria as a function of permafrost age (Kyr = thousands of years B.P.; Ma = millions of years B.P). GenBank accession numbers for sequences passing a bootstrap test of independent reproducibility AY390779–AY391120 and AY483124–AY483136.

The superior persistence of GP over GN bacterial DNA is in agreement with *in vitro* based predictions, but the superior persistence of DNA from non-spore-forming-GP Actinobacteria is surprising, because endospores are generally regarded as the hardiest of all cell types. Although endospores have special adaptations such as DNA binding α/β-type small acid soluble proteins (SASPs) to reduce the rate of genomic modification [11], they have no metabolic activity or active repair and their DNA will degrade with time. The mechanism behind the superior persistence of DNA from the non-spore-forming-GP Actinobacteria is currently unknown. Slow but continuous metabolic activity and DNA repair at subzero temperatures is one possibility [14]. Adaptations connected to dormancy might be another explanation [15]. Finally, the DNA could simply originate from dead bacteria, whose DNA for some reason, e.g., structural features, survived better. This has recently been shown for some parts of the human mitochondrial genome [16].

The observed rates of DNA degradation match theory [9], and indicate a limit for PCR amplifiable DNA between 400 kyr and 1.5 Ma, beyond which DNA is either severely crosslinked or non-detectable. Thus, our results represent the oldest reproducible and authenticated bacterial DNA to date. As cold conditions are critical for long-term DNA survival [8,12,17] the results strongly contradict claims of multi-Ma DNA sequences, or even putative viable cells of endospores and Proteobacteria, from non-frozen materials such as amber and halite [4–7]. None of these studies were confirmed by independent replication and/or measurements of DNA damage, and should be considered sceptically. Our results also contradict previous claims of the isolation of viable bacteria from many Ma old permafrost samples [2,3], which have also not been replicated independently [8]. Importantly, PCR based methods should be able to detect DNA from living bacteria, as well as the various dead and dormant cells, while less than 1% of the actual bacteria present are likely to be culturable [18].

The superior long-term DNA persistence of non-spore-forming GP bacteria has important implications for studies of cellular DNA preservation mechanisms, and for the search for extraterrestrial life in permafrost and ice deposits of Mars and Europa, where experiments have so far focused on the long-term survival of bacterial endospores under space conditions [19]. Our results suggest that these are probably not the most durable types of bacteria under frozen conditions.

References

1. Kennedy, M.J., Reader, S.L., and Swierczynski, L.M. (1994). Preservation records of microorganisms: evidence of the tenacity of life. Microbiology *140*, 2513–2529.
2. Shi, T., Reeves, R.H., Gilichinsky, D.A., and Friedmann, E.I. (1997). Characterization of viable bacteria in Siberian permafrost by 16S rDNA sequencing. Microb. Ecol. *33*, 169–179.
3. Vorobyova, E., Soina, V., Gorlenko, M., Minkovskaya, N., Zalinova, N., Mamukelashvili, A., Gilichinsky, D., Rivkina, E., and Vishnivetskaya, T. (1997). The deep cold biosphere: facts and hypotheses. FEMS Microbiol. Rev. *20*, 277–290.
4. Cano, R.J., and Borucki, M.K. (1995). Revival and identification of bacterial spores in 25- to 40-million year-old Dominican amber. Science *268*, 1060–1064.
5. 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.
6. Fish, S.A., Shepherd, T.J., McGenity, T.J., and Grant, W.D. (2002). Recovery of 16S ribosomal RNA gene fragments from ancient halite. Nature *417*, 432–436.
7. Vreeland, R.H., and Rosenzweig, W.D. (2002). The question of uniqueness of ancient bacteria. J. Ind. Microbiol. Biotechnol. *28*, 32–41.
8. Willerslev, E., Hansen, A.J., and Poinar, H. (2003). Isolation of nucleic acids and cultures from fossil ice and permafrost. Trends Ecol. Evol., in press.
9. Hofreiter, M., Serre, D., Poinar, H.N., Kuch, M., and Pääbo, S. (2001). Ancient DNA. Nat. Rev. Genet. *2*, 353–360.
10. Cooper, A., and Poinar, H.N. (2000). Ancient DNA: do it right or not at all. Science *289*, 1139.
11. Setlow, P. (1995). Mechanisms for the prevention of damage to DNA in spores of Bacillus species. Annu. Rev. Microbiol. *49*, 29–54.
12. 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.
13. Willerslev, E., Hansen, A.J., Brand, T., Binladen, J., Gilbert, T.M.P., Shaperio, B., Bunce, M., Wiuf, C., Gilichinsky, D.A., and Cooper, A. (2003). Diverse plant and animal DNA from Holocene and Pleistocene sedimentary records. Science *300*, 792–795.
14. Revkina, E.M., Friedmann, E.I., McCay, C.P., and Gilishinsky, D.A. (2000). Metabolic activity of permafrost bacteria below the freezing point. Appl. Environ. Microbiol. *66*, 3230–3233.
15. Mulyukin, A.L., Demkina, E.V., Kozlova, A.N., Soina, V.S., and El-Registan, G.I. (2001). Synthesis of anabiosis autoinducers by non-spore-forming bacteria as a mechanism regulating their activity in soil and subsoil sedimentary rocks. Microbiology *70*, 535–541.
16. Gilbert, M.T.P., Willerslev, E., Hansen, A.J., Barnes, I., Rudbeck, L., Lynnerup, N., and Cooper, A. (2003). Distribution patterns of post-mortem damage in human mitochondrial DNA. Am. J. Hum. Genet. *72*, 32–47.
17. Smith, C.I., Chamberlain, A.T., Riley, M.S., Cooper, A., Stringer, C.B., and Collins, M.J. (2001). Neanderthal DNA: not just old but old and cold? Nature *10*, 771–772.
18. Muyzer, G. (1998). Structure, function and dynamics of microbial communities. In Advances in Molecular Ecology. Carvalho, G.R. ed. (IOS Press, Amsterdam), pp. 87–117.
19. Nicholson, W.L., Munakata, N., Horneck, G., Melosh, H.J., and Setlow, P. (2000). Resistance of bacterial endospores to extreme terrestrial and extraterrestrial environments. Microbiol. Mol. Biol. Rev. *64*, 548–572.

[1]Department of Evolutionary Biology, Zoological Institute, University of Copenhagen, Universitetsparken 15, DK-2100, Denmark. *E-mail: ajhansen@zi.ku.dk [2]Henry Wellcome Ancient Biomolecules Centre, Department of Zoology, University of Oxford, Oxford OX1 3PS, UK. [3]Bioinformatics Research Center, University of Aarhus, DK-8000, Denmark [4]Soil Cryology Laboratory, Institute for PhysicoChemical and Biological Problems in Soil Science, Russian Academy of Sciences, 142290, Pushchino, Moscow Region, Russia. [5]Department of Carcinogenesis, The University of Texas, M.D. Anderson Cancer Center Science Park/Research Division, P.O. Box 389, Park Road 1C Smithville, Texas 78957, USA.