THE ROYAL SOCIETY

How microbial ancient DNA, found in association with human remains, can be interpreted

Franco Rollo and Isolina Marota

Dipartimento di Biologia Molecolare, Cellulare e Animale, Università di Camerino, I-62032 Camerino, Italy (*rollo@cambio.unicam.it*)

The analysis of the DNA of ancient micro-organisms in archaeological and palaeontological human remains can contribute to the understanding of issues as different as the spreading of a new disease, a mummification process or the effect of diets on historical human populations. The quest for this type of DNA, however, can represent a particularly demanding task. This is mainly due to the abundance and diffusion of bacteria, fungi, yeasts, algae and protozoans in the most diverse environments of the present-day biosphere and the resulting difficulty in distinguishing between ancient and modern DNA. Nevertheless, at least under some special circumstances, by using rigorous protocols, which include an archaeometric survey of the specimens and evaluation of the palaeoecological consistency of the results of DNA sequence analysis, glimpses of the composition of the original microbial flora (e.g. colonic flora) can be caught in ancient human remains. Potentials and pitfalls of this research field are illustrated by the results of research works performed on prehistoric, pre-Columbian and Renaissance human mummies.

Keywords: ancient DNA; molecular anthropology; prehistoric bacteria; palaeoethnology; Ice Man; human mummies

1. INTRODUCTION

A fascinating sector in the archaeomolecular field is the study of ancient micro-organisms: bacteria, filamentous fungi, yeasts, algae and protozoans. Because of the importance of the interactions of this type of micro-organism with humans, pathogens, such as *Mycobacterium tuberculosis* (Spigelman & Lemma 1993; Baron *et al.* 1996; Salo *et al.* 1994; Dixon *et al.* 1995; Taylor *et al.* 1996; Crubezy *et al.* 1997; Faerman *et al.* 1997), *Mycobacteriun leprae* (Rafi *et al.* 1994), *Yersina pestis* (Hummel *et al.* 1994) and *Treponema pallidum* (Rogan & Lentz 1995), have attracted most of the specialists' research efforts.

A widely employed experimental protocol for the archaeomolecular identification of a pathogenic bacterium is DNA extraction from desiccated soft tissue or bone, and polymerase chain reaction (PCR) amplification using PCR primer pairs (or sets of nested primer pairs) designed on the basis of highly variable regions of the chromosome of the relevant micro-organism. An example of this approach is given by the works of Salo *et al.* (1994) and of Faerman *et al.* (1997). The former started from a lung lesion of a spontaneously mummified body of an adult (40–45-year-old) female who died 1000 years ago in southern Peru. The tomb from which the body was exhumed was in a burial site (Chiribaya Alta) used by the Chiribaya, a mostly agricultural population that occupied the Lower Osmore Valley near the coastal community of Ilo. DNA was extracted from lesion tissue and enzymatically amplified using a primer pair designed to bind to a 123-bp long fragment of DNA which is part of a repetitive (10–16 copies per bacterial chromosome) insertion sequence (IS)-like element 1361 bp in length, called IS6110. The presence of this element in *M. tuberculosis* has been shown to correlate closely with clinically diagnosed tuberculosis.

In ancient Europe, tuberculosis was one of the most widely prevalent infectious diseases. Incidence in bone pathology in skeletal remains from medieval Lithuania suggests that 18–25% of the population suffered from it. Faerman *et al.* (1997) detected the presence of *M. tuberculosis* in skeletal remains from Lithuania, dated to the 15th–17th centuries, by amplifying a portion of the IS6110 element, and found that a much higher percentage of individuals was infected than had previously been thought.

For what concerns the important issue of the pathogenic eukaryotes (protozoans), Taylor *et al.* (1997) have developed a hemi-nested PCR method for amplifying *Plasmodium* nucleic acid. Their method detects all four malaria-producing *Plasmodium* species pathogenic to humans and was designed to amplify small fragments of DNA likely to remain in archaeological specimens. The method has been applied to two human ribs from separate individuals who had died almost 60 years previously from anaemia, thought to be due to malaria. *Plasmodium* DNA was confirmed in one of these cases; subsequently, sequencing of the PCR product identified the causative species as *Plasmodium falciparum*. It is important, however, to note that when the test was applied to the Granville mummy the result was negative. This mummy is of a female aged about 50 years from the site of Gurna (Egypt) (approximately 700 BC) and had previously been reported positive for *Plasmodium falciparum* using immunological methods.

There have also been a few attempts to perform a broad-range description of the ancient microbial DNA in a human mummy. We learned an important methodological lesson during the attempt (Marota *et al.* 1996) to give a molecular confirmation of a diagnosis of venereal syphilis for Maria of Aragon. Through the years, the Renaissance mummies of the abbey of San Domenico Maggiore in Naples (Fornaciari & Capasso 1996) have been submitted to accurate palaeopathological investigations which have allowed the researchers to discover two cases of infectious disease (smallpox and syphilis) and two of neoplastic pathology (skin epithelioma and colon adenocarinoma). One of the most interesting mummies of the San Domenico Maggiore abbey, is that of Maria of Aragon. Marchioness of Vasto and friend of Michelangiolo Buonarroti, Maria of Aragon (1503–1568) was a person of considerable importance in the Italian Renaissance. The first survey of her embalmed body, showed that the left arm was covered by a rectangular linen pocket filled with ivy leaves, and that this sort of dressing concealed an oval ulcer. A careful microscopical examination of the linen tissue showed that well-preserved necrobiotic leucocytes and bacterial cells of various shapes were still adhering to the fibres, in correspondence with the ulcer. For DNA extraction we used a small amount of desiccated cellular material, obtained by scraping the linen with a razor blade. DNA was PCR-amplified using primer pairs designed to bind to short fragments (respectively 95, 135 and 207 bp in length) of the 16S ribosomal rRNA gene of *Treponema pallidum*, the causative agent of venereal syphilis. Electrophoretic fractionation of the amplification products evidenced fluorescent bands of the expected length (figure 1). In addition, sequence analysis of the shorter (95 bp) fragment, using direct sequencing, showed approximately 85% base similarity with reference *T. pallidum* 16S rDNA. However, when the amplified DNA was cloned into a plasmid vector and sequence analysis was performed starting from individual clones (individual clones = amplicons), instead of being directly sequenced, no *T. pallidum* sequence was found. Rather, phylogenetic analyses suggested the presence of the genera *Propionibacterium*, *Mycobacterium*, *Peptostreptococcus*, *Clostridium* and *Capnocytophaga* (figure 2). An indirect confirmation of the diagnosis of venereal syphilis came, rather unexpectedly, when we read that Renaissance doctors usually prescribed 'salivation cures' to patients suffering from '*Morbus gallicus*'; hence a possible explanation for the finding of the DNA of members of the human oral cavity microbiota (*Capnocytophaga*, *Peptostreptococcus* and *Propionibacterium*) in the bandage (Marcotte & Lavoie 1998).

Pathogens, however, represent a minor fraction of the micro-organisms (mainly bacteria and fungi) interacting with humans or animals. We have, for example, a large variety of non-pathogenic microbial dwellers of the human skin, mouth and intestines. In the case of clothing made with natural materials such as plant fibres or animal skins, we can have micro-organisms saprobically living on them. Finally, at the death of the organism, either human or animal, the corpse (or the carcass) becomes a rich source of organic and inorganic nourishment for bacteria and fungi (putrefaction). A mummification process, either natural or artificial, acts by stopping

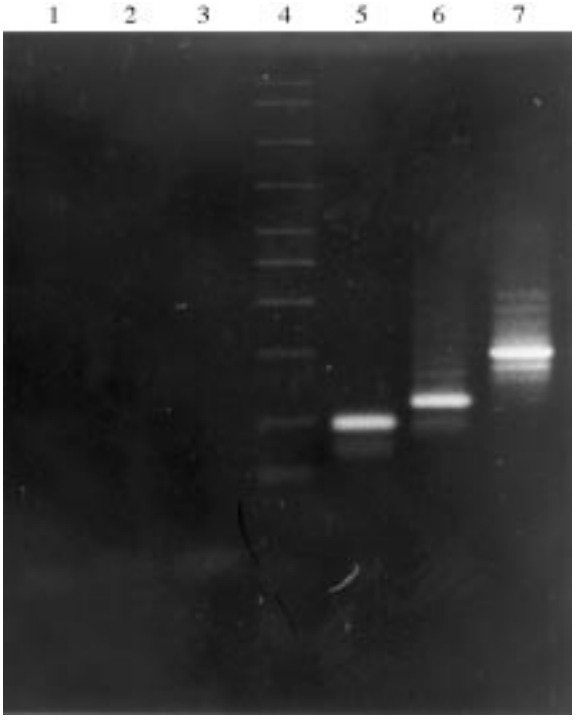

Figure 1. PCR amplification, using the amplification primer pairs Tp16s4f/Tp16s5r (95 bp), Tp16s1f/Tp16s2r (135 bp) and Tp16s1f/Tp16s3r (207 bp), of the DNA isolated from Maria of Aragon's arm band. 1, 2, extraction blanks; 3, reaction blank; 4, Bio-Rad's 50–2000 bp ladder; 5, DNA from the Tp16s4f/Tp16s5r band; 6, DNA from the Tp16s1f/Tp16s2r band; 7, DNA from the Tp16s1f/Tp16s3r band. About 200 mg of dry material scraped from the band were ground in a mortar with a pestle in the presence of 750 µl of an extraction medium containing 1% (w/v) SDS, 50 mM Na_2EDTA, 50 mM Tris-HCl (pH 8.0) and 6% (v/v) water-saturated phenol. The extraction medium was added stepwise to the sample (three aliquots of 250 µl each) and the resulting mixture was vortexed for 2 min, then centrifuged for 2 min (13 000 rpm) in a bench-top centrifuge. The supernatant was transferred in an Eppendorf tube and re-extracted a first time using 500 µl of phenol–chloroform–isoamilic alcohol (25/24/1) then chloroform and eventually (three times) using ether. As a final step, 40 µl of sodium acetate (2 M stock solution) and 1 ml ethanol were added to the aqueous phase and the suspension was kept at −20 °C for 12 h. The DNA was precipitated by centrifugation (13 000 rpm) for 10 min and the resulting pellet drained, vacuum desiccated for about 15 min and resuspendend in 40 µl distilled water. To eliminate the polymerase inhibitors, the DNA was purified by low-temperature agarose gel (2.5%) electrophoresis. The gel (10 cm × 6.5 cm × 0.4 cm) was run at 70 V for 1 h then stained with ethidium bromide and observed under UV light. The gel was cut into five fragments in correspondence with the DNA fluorescent smear and the blocks were stored at −25 °C until use. When needed, the agarose blocks were melted at 65 °C and 1 µl of the agarose–DNA suspension was directly added to a reaction mixture (50 µl) containing 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1 mg ml^{-1} gelatin, 200 mM each of dATP, dCTP, dTTP and dGTP, 300 ng of each oligonucleotide primer, and 2.5 units of *Taq* polymerase. The thermal cycler was set as follows (40 cycles): 94 °C for 7 min (initial denaturation); 94 °C for 30 s (denaturation); 50 °C for 30 s (annealing); 72 °C for 1 min (elongation); 72 °C for 10 min (final elongation). Amplification blanks were obtained by the use of agarose blocks cut outside the DNA smear.

the proliferation of the cadaveric flora and thus allows the preservation of the soft tissues. We must bear in mind, however, that whatever the nature of the mummification process, this will act by preserving the soft tissues, but also the microbial cells already present in them.

The Tyrolean Ice Man, or Ötzi, offers an example of how a controversial taphonomical history can furnish interesting hints for the study of ancient micro-organisms. The so-called Ice Man is a mummified human body found in an Alpine glacier on 19 September 1991 at 3270 m above sea level. The most relevant feature of the find (radiocarbon dated to 3350–3100 BC, corresponding to Late Neolithic) is the amazing state of preservation of the mummy and of the clothing and equipment found on the body and near it; they include, among many other items, extremely perishable ones, such as a large fragment of a cloak made of knotted tufts of grass, a fur hat and a wooden bow with arrows and quiver (Spindler 1995).

The Ice Man lay in a chamber-like depression below a rocky ledge, sheltered from the shearing flow of glacial ice. So trapped, the corpse was not expelled with the regular glacial turnover. In the course of the years following the discovery, archaeologists and anthropologists have made several speculations on the process of mummification responsible for the preservation of the Ice Man's body. Until 1995, the prevailing one was that the corpse had undergone rapid dehydration by a warm wind (an autumn *föhn*) and been subsequently covered by snow. An alternative speculation was that the body and equipment became rapidly frozen, and were then covered by a porous layer of snow which allowed the body to air desiccate (Spindler 1995). Bahn (1996), on the other hand, suggested that the corpse was preserved in the same way as the many frozen carcasses of mammoths and other Ice Age animals in Siberia and Alaska. They were preserved by the build-up of ice in the sediments that enveloped the bodies: the ice layers desiccated the soil and dehydrated the carcasses. Unlike freeze-drying, where the original form remains intact, this process shrivels the body.

Neither of these hypotheses, however, can entirely account for a number of features which are being progressively revealed by the investigations carried out independently in several laboratories since the first exploration of the Ice Man's site. For example, histological and biochemical analyses (Bereuter *et al*. 1997) have shown an almost complete loss of the Ice Man's epidermis, accompanied by profound post-mortem alterations of skin triacylglycerols, which imply a prolonged (up to several months) immersion in water before dry weather and, possibly, warm winds desiccated the corpse. This result, in turn, stimulated interest in the results of microbiological analyses previously performed on the Ice Man's grass clothing (Rollo *et al*. 1995*a*,*b*, 1997) describing the finding of algal (chrysophicean) cysts on the Neolithic grass and indicating that the largest fraction of the DNA that was extracted from the grass was of algal origin.

Rollo *et al*. (1994, 1995*a*,*b*) and Ubaldi *et al*. (1996) also analysed the fungal hyphae that cover the Ice Man's grass clothing. With this in view, they extracted DNA from samples of grass from the clothing, and the DNA was then PCR-amplified using, respectively, primer pairs specific for the region containing the internal transcribed spacers and the 5.8S rDNA (ITS) and primer pairs specific for an approximately 600-bp long fragment of the nuclear small-subunit ribosomal DNA (SSU rDNA) repeat units of eukaryotes. The amplification products were cloned and sequenced. Sequence analysis of 20 ITS and 10 SSU rDNA amplicons indicated that three types of fungal DNA were associated with the Neolithic grass. Phylogenetic analyses, using 5.8S and SSU rDNA fungal reference sequences from databases showed that the DNAs came, respectively, from a basidiomycete, phylogenetically close to *Leucosporidium scottii*, a psycrophilic yeast-like micro-organism isolated from, among other substrates, soil, plant material and water in Antartica and Canada, and from two ascomycetes, one of which is possibly related to the Eurotiales.

As a conclusion to this introductory review, we would like to cite the possibility of using the analysis of bacterial and fungal DNA as a molecular test for archaeological bone preservation. The principle of this test is that if the bone histology is preserved, one should detect very little or no microbial DNA. On the other hand, if the bone preservation is poor, sediments and hence bacteria and fungi have entered the tissue. Figure 3 shows the results of the PCR amplification, using a 'universal' bacterial primer pair (Lane 1991), of the DNA extracted from Etruscan bone specimens coming from two archaeological sites characterized by different geopedological conditions. One can note that the group of specimens on the left side of the gel, on average, gives much stronger amplification signals than the group on the right, in accordance with the more favourable conditions of bone preservation of the second site. However, while this analysis seems straightforward, attempts to correlate the presence–absence of bacterial DNA in the bone with poorer or better preservation of the original human DNA have given contradictory results, as shown by Vernesi *et al*. (1998). Further experimentation is thus required to assess the real effectiveness of microbial DNA as a diagenetic marker.

2. MOLECULAR MICROBIAL PALAEOECOLOGY OF THE HUMAN BODY

(a) *Palaeoecological consistency and the 'fresco' model*

Attempts to molecularly identify ancient micro-organisms which are not obligate parasites of humans or animals are hampered by the difficulty of distinguishing between the DNA of truly ancient saprobes or opportunists and the DNA of soil micro-organisms that may have colonized the remains in more recent times. The restoration of the embalmed body of King Ramses II (XIX Dynasty, 1290–1224 BC) performed in Paris in the years 1976–77 provides an eloquent example of the level of infiltration reached by modern microbial (fungal) contaminants. When tissue samples taken form the pharaoh's mummy were plated, 370 colonies, representative of 89 fungal species, were isolated (Mouchacca 1985).

To overcome the issue of distinguishing between ancient and modern micro-organisms, Rollo *et al*. (1997) have proposed the application of a palaeoecological consistency criterion. Briefly, one should check whether the putative ecophysiology and distribution of the micro-organisms identified on the basis of DNA sequencing is consistent with the present environmental characteristics of the site from which the specimen comes or, rather, the

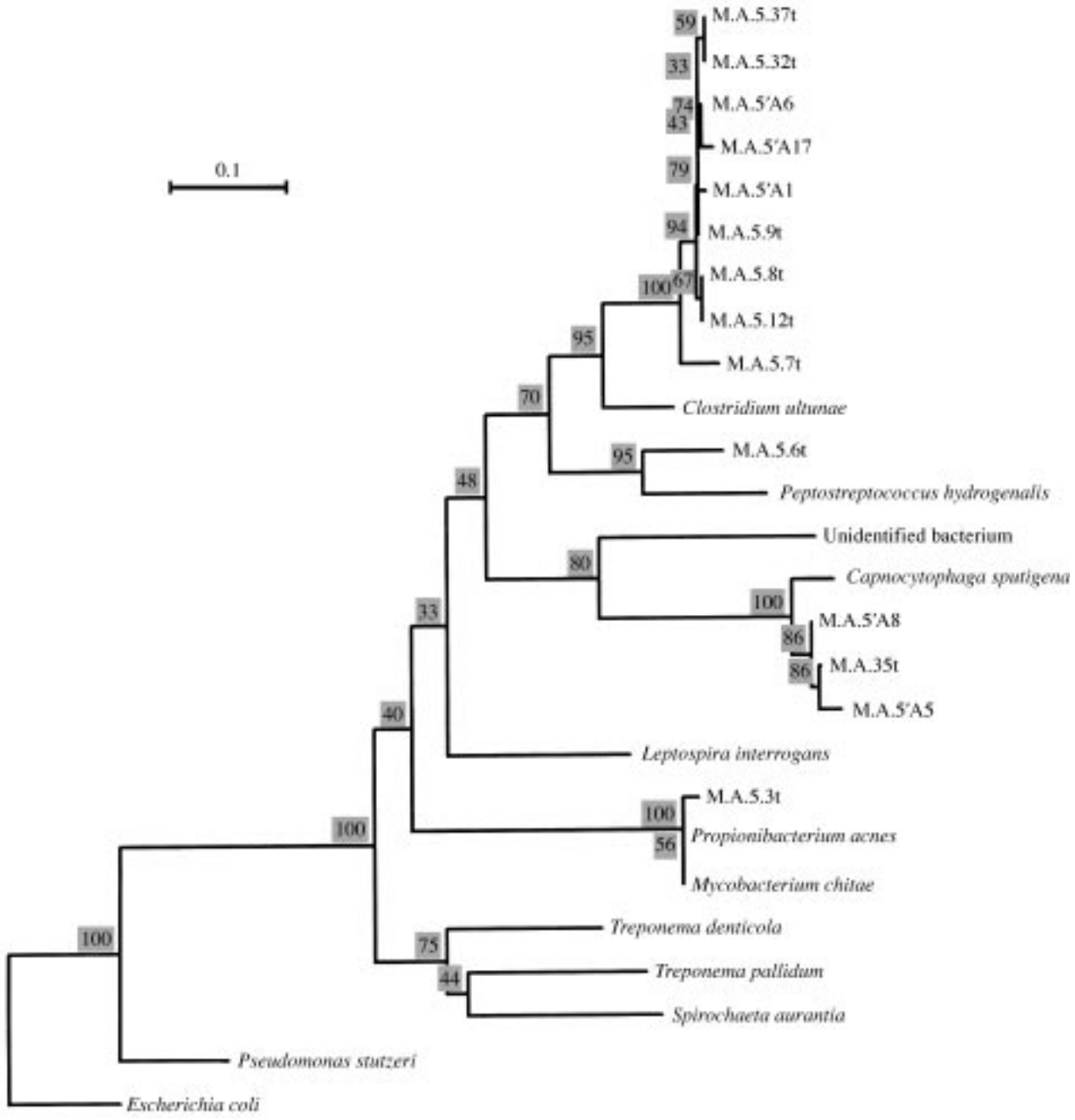

Figure 2. 16S rDNA bootstrapped distance tree of the eubacteria, inclusive of the nucleotide sequences of the bacteria detected in Maria of Aragon's arm band. Comparisons with reference sequences in GenBank and in the EMBL data library were performed using the BLAST (Altschul *et al*. 1990) search program. To establish phylogenetic correlations among ancient and modern bacterial species the 16S rDNA nucleotide sequences were aligned using the CLUSTAL V function. The tree was constructed using the TREECON (Van de Peer & De Wachter 1993) program. In particular, the distances were corrected according to Tajima & Nei (1984) and the dendrogram was calculated using the neighbor-joining method (Saitou & Nei 1987).

information stemming from DNA analysis better fits with the traces left by the microbial dwellers of a living body. It is important to note that in the case of ubiquitarian micro-organisms, e.g. certain species of clostridia, it will be extremely difficult, if not impossible, to determine for each individual sequence whether it comes from an ancient or a modern micro-organism. However, the sequences as a whole will provide some information (Rollo 1998). This concept is best exemplified by a poorly preserved fresco, e.g. the world famous Leonardo da Vinci Last Supper, in the church of Santa Maria delle Grazie, Milan. If you look at the wall from close up, you just see stains which may be paint or may be damp. However, if you keep sufficiently far away, you will be able to appreciate the beauty of Leonardo's masterpiece.

(b) *The original flora of the colon of a pre-Columbian mummy*

It is now recognized that all external body surfaces have a normal resident bacterial flora, and this includes

the digestive tract. Because of cell turnover, gut surfaces are coated with dead and desquamating cells, and these provide an excellent basal nutrient source, to which can be added nutrients passing through the lumen and the gut (Hill 1995). The bacterial flora represents a not minoritarian component of the complex intestinal ecosystem. Its distribution along the intestinal tract is, however, highly variable in number and composition and can be influenced by diet, interaction among the different groups of micro-organisms, drugs assumption, toxins and carcinogenic substances. Under normal conditions the upper part of the small bowel (duodenum, jejunum) is considered to have a low microbial content, while its lower portion (ileum) is characterized by higher cell numbers. The highest microbial content is found in the large bowel (colon).

To investigate the possibility of collecting data on the composition of the bacterial flora of the colon for historical human populations, Ubaldi *et al.* (1998) studied a pre-Columbian mummy. The mummy, presently at the National Museum of Anthropology and Ethnology of Florence, Italy, is of a young woman aged 20 ± 3 years and is known to come from Cuzco (Peru), the ancient capital of the Inca kingdom. The archaeological dating of this body has been the subject of repeated revisions in the course of time. In the past decade, on the basis of the funerary goods, the date was assumed to be 15th–16th century AD. This figure was subsequently moved back to 14th century AD. Radiocarbon analyses recently performed on a group of Andean mummies conserved at the 'L. Pigorini' Prehistoric and Ethnographic Museum (Rome, Italy), however, suggest that a more reliable date might be 10th–11th century AD (R. Machiarelli, personal communication). A few years ago the Andean mummy (a natural mummy) was the subject of a necropsy performed by Fornaciari *et al.* (1992). The dissection of the body showed that the internal organs, stomach, lungs, intestine, liver, heart, etc. are well preserved.

As a first step, DNA was phenol-extracted from samples of oesophagus, stomach, pylorus, small intestine, ascending colon, transverse colon, descending colon, liver, (left and right) lung, diaphragm, pericardium, myocardium and aorta of the mummy (14 in total). The DNA fractions were then analysed by agarose gel electrophoresis. All the extracts were shown to contain short DNA fragments ranging in length from a few dozen base pairs to approximately 200 bp.

The degree of conservation of the original mummy DNA was determined using two experimental approaches: evaluation of the extent of racemization of aspartic acid and quantification of mtDNA copy number. The estimation of aspartic acid racemization level (performed on myocardic and colon tissue samples) provided a value for the D:L ratio fully comparable (Poinar *et al.* 1996) with the ratio shown by fresh amino-acid preparations (0.035). This result is in principle, compatible with the preservation of the original mummy DNA.

Subsequently, to measure mtDNA copy number, a competitive PCR system according to Förster (1994) was employed. This system is based on the amplification of a short (103 bp) tract of the hypervariable region I of the mtDNA control region in the presence of a competitor DNA of 81 bp in length. A commonly experienced aspect of PCR is the low reproducibility level of the amount of product yield, even under the most controlled assay conditions. This variability may depend on different causes, including machine performance, reaction conditions, presence of inhibitors, and differences in sample preparation and purification of nucleic acids (Clementi *et al.* 1993). A reliable approach to molecular quantitation using PCR amplification is that based on co-amplification of two similar templates (the target sequence and the reference template introduced at a known amount) of equal or similar length sharing the primer recognition sequences. During amplification, the two templates compete for the same primer set (competitive PCR) and consequently amplify at the same rate independently of the number of PCR cycles and of any predictable or unpredictable variable influencing the PCR amplification. The analysis of DNA preparations from mummy tissue samples showed that all contained human mtDNA though at a different multiplicity (table 1).

Table 1. *Human mitochondrial DNA and bacterial 16S rDNA (fragment-length = 103 and 196 bp, respectively) copy number (per reaction mixture) in different tissue samples from an Andean mummy*

tissue or organ	mtDNA	16S rDNA
oesophagus	100	n.t.
transverse colon	100	3000
myocardium	200	n.t.
aorta	600	n.t.

n.t. = not tested.

At this point, as both tests convincingly indicated that the original mummy DNA was preserved, the quest for bacterial DNA could be initiated. The first step was to determine the distribution of the bacterial DNA in the different organs and tissues of the mummy. With this in view, all the DNA preparations were PCR-amplified using a primer pair (29f/98r) designed to bind to a very short portion (approximately 100 bp) of the bacterial 16S rDNA according to the principle of the so-called 'consensus sequence PCR' (Relman 1993). Different samples produced amplification signals of a different intensity. In particular, liver, lungs and diaphragm produced no signal or very weak signals. On the other hand, ascending, transverse and descending colon produced very strong signals while weak signals were produced by pylorus and the small intestine. The abundance of bacterial DNA in the large bowel of the mummy compared to the relatively poor content of the small bowel is best explained by the hypothesis that the desiccation process responsible for the mummification of the body has also preserved the intestinal flora (figure 4).

In this case too, the variations in bacterial DNA copy number in the mummy's small and large bowel were assessed in a more precise manner by the use of competitive PCR. In this case the competitor was obtained from a cloned fragment of 16S rDNA gene (table 1). Competitive PCR can also be used to estimate the relative proportions of intact ('modern') and degraded ('ancient') bacterial templates in the different organs of the mummy.

To identify the bacteria, the 16S rDNA amplification products of the transverse colon sample were cloned into

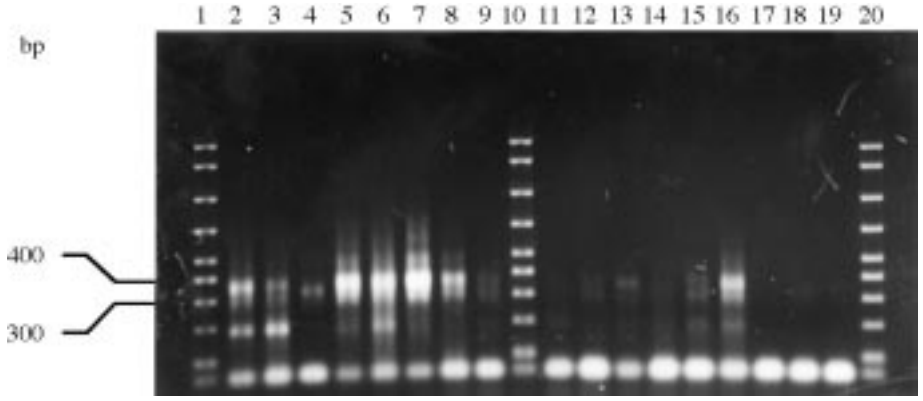

Figure 3. PCR amplification using bacterial 16S rDNA universal primers (27f/342r) of the DNA extracted from two sets of human bones from the Etruscan necropolis of Magliano Cancellone (7th–6th century BC). 2–9, Magliano Cancellone I; 11–16, Magliano Cancellone II/III; 17–19, extraction blanks; 1, 10, 20 molecular-size marker (Bio-Rad, AmpliSize). DNA extraction and PCR amplification conditions were as reported in Vernesi *et al*. (1998).

a pMOSBlue plasmid vector and the nucleotide sequence of 15 amplicons was determined. The sequences were then used to scan the European Molecular Biology Laboratory (EMBL) and GenBank data libraries. Eleven out of the 15 sequences considered were identified as *Clostridium botulinum*, an organism commonly isolated from soil, marine and lake sediments. *C. botulinum* is also found in animal, bird and fish intestines, and in food. The remaining sequences belong to the *Clostridium* sp. *C. algidicarnis* and *Eubacterium pectinii*. One could take this rather surprising result as an indication that the Andean woman was fed with honey before death as *C. botulinum* spores are frequently found in natural honey. We can observe, however, that all these identifications should be taken cautiously due to the shortness (approximately 60 bp) of the sequences compared and to the low level of similarity with the reference sequences displayed by several of them.

To obtain a more convincing inference, the DNA preparation from the transverse colon was PCR-amplified using a different primer pair (338f/531r) designed to bind to a longer (196-bp long) portion of the 16S rDNA, and the amplification products were cloned and sequenced. This time, the taxon composition was very different from the previous one; *C. algidicarnis* was the predominant species, followed in relative abundance by *C. cochlearium*, *C. aurantibutyricum* and *C. intestinalis*. This demonstrates that the increase of the target 16S rDNA sequence up to 196 bp has allowed an acceptable identification to be performed while keeping within the size range of the DNA fragments extracted from the mummy tissues and, in general, within a size range compatible with most of the ancient DNA investigations.

One may ask why only 16S rDNA sequences from clostridia are found. There are several possible explanations for this phenomenon. The first is that they are extremely abundant representatives of the anaerobic gut flora. Their proportion is most probably underestimated by the traditional cultivation assays. Second, the clostridia are endospore-forming bacteria and it is known that the spore offers an excellent protection to the DNA (Setlow 1992). There is the further possibility of an amplification bias (Suzuki & Giovannoni 1996). This, however, seems unlikely, as two different sets of primers have both given consistent results at least for what concerns taxon identification at the genus level. Finally, the prevalence of clostridia might reflect a pathological state of the colon of this particular mummy.

(c) *Microbial palaeoecology of the Tyrolean Ice Man*

A study where the results have been intepreted according to a criterion of palaeoecological consistency has been performed very recently (Cano *et al*. 1999). To check whether over 5000 years of permanence under the glacier have totally cancelled the traces of the bacteria that dwelled in the gastrointestinal tract of the neolithic herdsman–hunter or whether some remains of prehistoric bacteria were left, we selected three types of samples: the first was represented by grass fragments from the boots and the cloak, while the second and the third by biopsies from the Ice Man's stomach and colon, respectively. DNA was extracted from the three groups of samples and PCR-amplified using universal primer pairs targeted to portions of the 16S rDNA. Amplification products were cloned and amplicons from the three groups of libraries sequenced, and the sequences were used for database scanning. The results showed that the bacterial flora associated with the grass clothing included, among others, species belonging to the genera *Zoogloea*, *Curtobacterium*, *Arthrobacter, Desulfitobacterium* and *Sphingobacterium*. On the other hand, bacterial sequences obtained from stomach biopsies were few in diversity and consisted mainly of *Burkholderia* spp. and *Pseudomonas* spp. Finally, the colon showed a wide array of *Clostridium* and *Eubacterium*, most of which are recognized common inhabitants of the human intestines, and *Vibrio*. The strong differences found in the composition of the bacterial flora of the grass clothing (possibly representative of the microbiological situation of the glacier), stomach and colon, clearly shows that the body has been colonized by micro-organisms from the external environment only to a limited extent during its long taphonomical history. This result is consistent with the demonstration given by Handt *et al*. (1994) that fragments of the original mtDNA of the mummy are left.

Very recently, we have undertaken the analysis of microbial DNA in samples of the Ice Man's skin and muscle. In general, during the first stages of putrefaction, a dead body is attacked from the inside by the anaerobic or microaerophilic flora of the intestines, and from the outside by opportunistic saprobes present in the environment where the body lies. For a better understanding of the taphonomical history of the Ice Man, it would be of the greatest interest to analyse bacterial and, in general, microbial DNA sequences from skin and muscle specimens. Unfortunately, however, the mummy was swabbed with phenol the day following its recovery from the

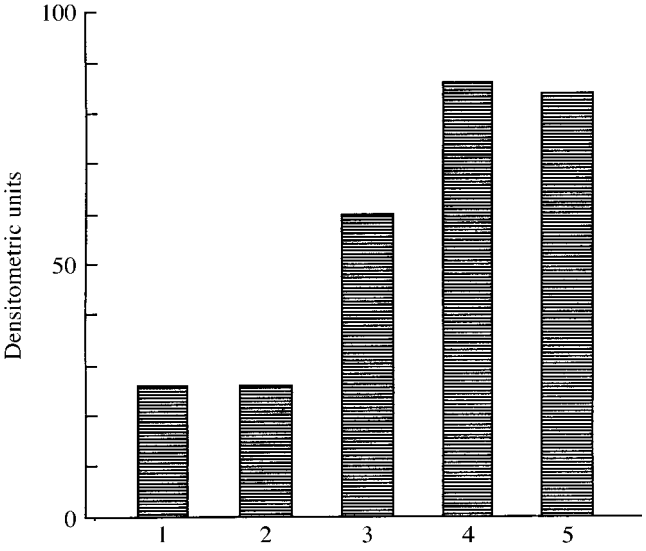

Figure 4. Densitometric analysis of the bands produced following PCR amplification using the 'universal' bacterial 16S rDNA primer pair 27f/98r of the DNA isolated from tissue samples of the gastrointestinal tract of an Andean mummy: 1, pylorus; 2, small intestine; 3, ascending colon; 4, transverse colon; 5, descending colon.

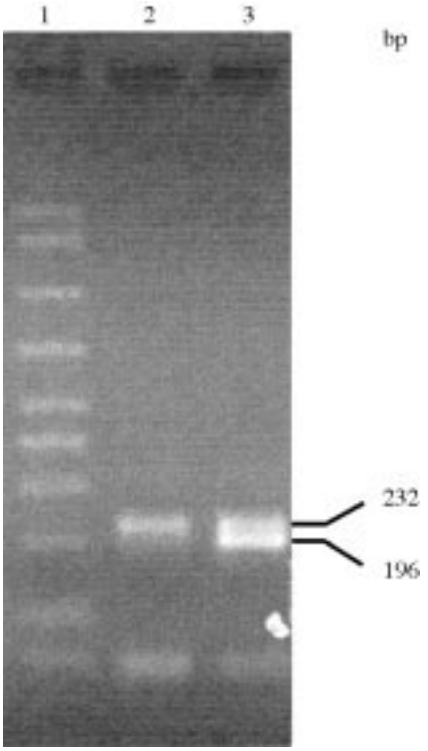

Figure 5. Quantification of low-molecular-weight (196 bp) bacterial 16S rDNA copy number in phenol-treated and untreated Ice Man's skin samples using competitive PCR. Both reaction mixtures contained 1000 copies of competitor. 1, Bio-Rad's 50–2000 bp ladder; 2, treated skin; 3, untreated skin. Enzymatic amplifications were performed in 50 µl of a reaction mixture containing 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1 mg ml^{-1} gelatin, 200 mM (each) dNTPs, 300 ng of each oligonucleotide primer (338f: 5′-AACTGAGACACGGTCCAGAC-3′; 531r: 5′-ACGCTTG-CACCCTCCGTATT-3′), 2.5 units *Taq* polymerase, 1 µl DNA from the Ice Man's skin and 1000 molecules of competitor. Following an initial step at 94 °C for 5 min, the thermal cycler was set as follows: 94 °C for 60 s (denaturation); 30 s at 56 °C (primers annealing); 72 °C for 60 s (elongation). Forty amplification cycles were performed. To eliminate small amounts of *Eschericia coli* DNA possibly present in the commercial enzyme preparations, the *Taq* polymerase, and the reaction components were pre-treated with 3 units of DNase I (from bovine pancreas) for 30 min at room temperature. After incubation, the DNase I was inactivated by boiling for 10 min at 94 °C. As a competitor, we employed a fragment of *Variovorax paradoxus* 16S rDNA which had been PCR-amplified using the 338f/531r primer pair (total length 232 bp) and then cloned into a pMOSBlue vector.

glacier (24 September 1991). If any trace of ancient microbial colonization of the skin was left, this has most probably been removed by the treatment. It was therefore fortunate that, during the very first attempts to pull the mummy out of the ice, actuated with the help of a pneumatic chisel, the left buttock and thigh underwent severe soft tissue damage with consequent release of skin, muscle and blood vessel portions into the snow. Several tissue specimens, which had escaped the phenol treatment, could be recovered and identified (Capasso *et al.* 1995) during the archaeological expedition of 1992.

Figure 5 shows the result of a competitive PCR assay designed to test the relative abundance of low-molecular-weight bacterial DNA in phenol-treated and untreated Ice Man's skin fragments. One can appreciate the dramatic difference between the two specimens. The phenol treatment has clearly removed most of the bacterial DNA originally present on the glacial mummy's skin. The untreated specimen, on the other hand, is rather rich in low-molecular-weight bacterial DNA; a similar result has been obtained in the case of a muscle fragment from the Ice Man's left thigh. At the moment, we are screening 16S rDNA amplicon libraries from these two specimens.

3. CONCLUSIONS AND PERSPECTIVES

According to a recent school of thought, no entirely convincing authentication can be performed on the sole basis of a nucleotide sequence retrieved from an ancient sample. Rather, sequence analysis should be the last link in a long chain of assays whose function is to guarantee that the original sample DNA is indeed preserved.

The use of DNA technology to describe ancient microbiota in human mummies undoubtedly poses a number of problems, mainly due to the diffusion of modern microorganisms in the most diverse environments. Despite these problems, thanks to the application of archaeometric approaches and of criteria of palaeoecological consistency, it seems possible, at least under some special circumstances, to catch a glimpse of the composition of the original microbial flora of an ancient human or animal body

As a stimulating non-human perspective, we may note that a relatively high rate of success has been reported for the analysis of mtDNA in permafrost-preserved Siberian mammoths and this despite the relevant age of the specimens. Hagelberg *et al.* (1994) extracted DNA from bones of the Khatanga mammoth. This consisted of the partial carcass of an adult male excavated in 1977 from alluvial sand in the eastern Taimyr peninsula. The age of the specimen was established to be at least 47 000 years by accelerator mass spectrometry (AMS) radiocarbon dating.

Höss *et al.* (1994) extracted DNA from the soft tissues of five different mammoths varying in age from 9700 to more than 50 000 years. Enzymatic amplification of a 93 bp fragment of the mitochondrial 16S rRNA gene yielded an amplification product from four of the five individuals, and specifically from 7 out of the 15 extractions performed. More recently, similar results have been obtained in the case of the Enmynveyem mammoth by Derenko *et al.* (1997). This mammoth was discovered in 1986 in the Enmynveyem River Valley of the Chukotka Peninsula of north-eastern Siberia. The remains, radiocarbon dated to $32\,850 \pm 900$ years BP, consist of intact portions of the right hind femur, tibia and fibula with articulated muscle and skin.

On the basis of the above cited results and of what we presently know on microbial DNA preservation in mummified bodies, we feel entitled to think of the Siberian mammoths as attractive candidates for future palaeomicrobiological investigations.

This work was funded by the Italian Ministry for the University and the Scientific Research (M.U.R.S.T.), local funds and Italian National Research Council 'Beni Culturali' project, grant no. 97.00611.PF36.

REFERENCES

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. 1990 Basic local alignment search tool. *J. Molec. Biol.* **215**, 403–410.

Bahn, P. G. 1996 *Tombs, graves and mummies*. New York: Barnes & Noble.

Baron, H., Hummel, S. & Herrmann, B. 1996 *Mycobacterium tuberculosis* complex in ancient human bones. *J. Archaeol. Sci.* **23**, 667–671.

Bereuter, T. L., Mikena, T. & Reiter, C. 1997 Iceman's mummification—implications from infrared spectroscopical and histological studies. *Chem. Eur. J.* **3**, 1032–1038.

Cano, R. J., Tiefenbrunner, F., Ubaldi, M., del Cueto, C., Luciani, S., Cox, T., Orkand, P., Kunzel, K. H. & Rollo, F. 1999 Molecular bacteriology of the neolithic glacier mummy from the Alps. (In preparation.)

Capasso, L., Capelli, A., Dal Rì, L., Frati, L., Mariani-Costantini, R. & Nothdurfter, H. 1995 I resti umani dell'Hauslabjoch raccolti durante la campagna di scavo del 1992: rapporto preliminare. In *Der Mann im Eis* (ed. K. Spindler, E. Rastbichler-Zissernig, H. Wilfing, D. zur Nedden & H. Nothdurfter), pp. 241–245. Wien, New York: Springer.

Clementi, M., Menzo, S., Bagnarelli, P., Manzin, A., Valenza, A. & Varaldo, P. E. 1993 Quantitative PCR and RT-PCR in virology. *PCR Meth. Applic.* **2**, 191–196.

Cruberèzy, E., Poveda, J. D., Montagnon, D. & Ludes, B. 1997 Identification of *Mycobacterium tuberculosis* or *bovis* DNA in Egyptian Pott's disease of a 5400 year old. In *Ancient DNA IV* (Conference Abstracts), Gottingen, 5–7 June 1997.

Derenko, M., Malyarchuk, B. & Shields, G. F. 1997 Mitochondrial cytochrome *b* sequence from a 33 000 year-old woolly mammoth (*Mammuthus primigenius*). *Ancient Biomolecules* **1**, 149–153.

Dixon, R. A., Aveling, E. & Roberts, C. A. 1995 Detection of a *Mycobacterium tuberculosis* specific insertion element from ancient human rib using the polymerase chain reaction. In *Ancient DNA III* (Conference Abstracts), Oxford, 20–22 July 1995.

Faerman, M., Jankauskas, R., Gorski, A., Bercovier, H. & Greenblatt, C. L. 1997 Prevalence of human tuberculosis in a medieval population of Lithuania studied by ancient DNA analysis. *Ancient Biomolecules* **1**, 205–214.

Fornaciari, G. & Capasso, L. 1996 Italian mummies. In *Human mummies. A global survey of their status and the techniques of conservation* (ed. K. Spindler, E. Rastbichler-Zissernig, H. Wilfing, D. zur Nedden & H. Nothdurfter), pp. 195–203. Wien, New York: Springer.

Fornaciari, G., Castagna, M., Viacava, P., Tognetti, A., Esteva, M., Segura, E. L. & Bevilacqua, G. 1992 Malattia di Chagas in una mummia peruviana Inca del Museo Nazionale di Antropologia ed Etnologia di Firenze. *Arch. Antrop. Etnol.* **122**, 369–376.

Förster, E. 1994 An improved general method to generate internal standards for competitive PCR. *Biotechniques* **16**, 18–20.

Hagelberg, E., Thomas, M. G., Cook, C. E., Sher, A. V., Baryshnikov, G. F. & Lister, A. M. 1994 DNA from ancient mammoth bones. *Nature* **370**, 333–334.

Handt, O. (and 12 others) 1994 Molecular genetic analyses of the Tyrolean Ice Man. *Science* **264**, 1775–1778.

Handt, O., Krings, M., Ward, R. H. & Paabo, S. 1996 The retrieval of ancient human DNA sequences. *Am. J. Hum. Genet.* **59**, 368–376.

Hill, M. J. 1995 The normal gut bacterial flora. In *Role of gut bacteria in human toxicology & pharmacology* (ed. M. J. Hill), pp. 3–17. London: Taylor & Francis.

Höss, M., Pääbo, S. & Vereshchagin, N. K. 1994 Mammoth DNA sequences. *Nature* **370**, 333.

Hummel, S., Menzel, A., Uy, A. *et al.* 1994 DNA analysis of inherited and infectious disease-perspective for paleopathology. In *Xth European Meeting of the Paleopathology Association*, 29 August–3 September, Göttingen.

Lane, D. 1991 16S/23S rRNA sequencing. In *Nucleic acids techniques in bacterial systematics* (ed. E. Stackebrandt & M. Goodfellow), pp. 115–173. Chichester, UK: Wiley.

Marcotte, H. & Lavoie, M. C. 1998 Oral microbial ecology and the role of salivary immunoglobulin A. *Microbiol. Molec. Biol. Rev.* **62**, 71–109.

Marota, I., Fornaciari, G. & Rollo, F. 1996 La sifilide nel rinascimento: identificazione di sequenze ribosomali batteriche nel DNA isolato dalla mummia di Maria d'Aragona (XVI secolo). *Antropologia Contemporanea* **19**, 157–174.

Mouchacca, J. 1985 Les champignons. In *La momie de Ramses II* (ed. D. L. Balout & C. Roubet), pp. 119–152. Paris: Editions Recherches sur les Civilisations.

Poinar, H. N., Höss, M., Bada, J. L. & Paabo, S. 1996 Amino acid racemization and the preservation of ancient DNA. *Science* **272**, 864–866.

Rafi, A., Spigelman, M., Stanford, J., Lemma, E., Donoghue, H. & Zias, J. 1994 *Mycobacterium leprae* DNA from ancient bone detected by PCR. *Lancet* **343**, 1360–1361.

Relman, D. A. 1993 The identification of uncultured microbial pathogens. *J. Infect. Dis.* **168**, 1–8.

Rogan, P. K. & Lentz, S. 1995 Molecular genetics evidence suggesting treponematosis in pre-columbian Chilean mummies (Conference Abstract). In *II World Congress on Mummy Studies*, Universidad di los Andes, Instituto Colombiano di Antropologia.

Rollo, F. 1998 Ancient DNA: problems and perspectives for molecular microbial palaeoecology. In *Advances in molecular ecology* (ed. G. Caravalho), Nato Science Series. Amsterdam: IOS Press. (In the press.)

Rollo, F., Asci, W., Antonini, S. & Ubaldi, M. 1994 Molecular ecology of a neolithic meadow, the DNA of the grass remains from the archaeological site of the Tyrolean Iceman. *Experientia* **50**, 576–584.

Rollo, F., Asci, W., Marota, I. & Sassaroli, S. 1995*a* DNA analysis of grass remains found at the iceman's archaeological site. In *Der Mann im Eis* (ed. K. Spindler, E. Rastbichler-Zissernig, H. Wilfing, D. zur Nedden & H. Nothdurfter), pp. 91–105. Wien, New York: Springer.

Rollo, F., Sassaroli, S. & Ubaldi, M. 1995*b* Molecular phylogeny of the fungi of the Iceman's grass clothing. *Curr. Genet.* **28**, 289–297.

Rollo, F., Luciani, S. & Ubaldi, M. 1997 Ancient micro-organisms offer new clues to the Iceman's mummification. *Hum. Evol.* **12**, 197–208.

Saitou, N. & Nei, M. 1987 The neighbor-joining method, a new method for reconstructing phylogenetic trees. *Molec. Biol. Evol.* **4**, 406–425.

Salo, W. M., Aufderheide, A. C., Buikstra, J. & Holcomb, T. 1994 Identification of *Mycobacterium tuberculosis* DNA in a pre-Columbian Peruvian mummy. *Proc. Natn. Acad. Sci. USA* **91**, 2091–2094.

Setlow, P. 1992 I will survive: protecting and repairing spore DNA. *J. Bacteriol.* **174**, 2737–2741.

Spigelman, M. & Lemma, E. 1993 The use of the polymerase chain reaction to detect *Mycobacterium tuberculosis* in ancient skeletons. *Int. J. Osteoarcheol.* **3**, 137–143.

Spindler, K. 1995 *The man in the ice.* London: Phoenix.

Suzuki, M. T. & Giovannoni, S. J. 1996 Bias caused by template annealing in the amplification of mixture of 16S rRNA genes by PCR. *Appl. Environ. Microbiol.* **62**, 625–630.

Tajima, F. & Nei, M. 1984 Estimation of evolutionary distance between nucleotide sequences. *Molec. Biol. Evol.* **1**, 269–285.

Taylor, M. G., Crossey, M., Saldanha, J. & Waldron, T. 1996 DNA from *Mycobacterium tuberculosis* identified in medieval human skeletal remains using polymerase chain reaction. *J. Archaeol. Sci.* **23**, 789–798.

Taylor, G. M., Rutland, P. & Molleson, T. 1997 A sensitive polymerase chain reaction method for the detection of *Plasmodium* species DNA in ancient human remains. *Ancient Biomolecules* **1**, 193–203.

Ubaldi, M., Sassaroli, S. & Rollo, F. 1996 Ribosomal DNA analysis of culturable deuteromycetes from the iceman's hay: comparison of living and mummified fungi. *Ancient Biomolecules* **1**, 35–42.

Ubaldi, M., Luciani, S., Marota, I., Fornaciari, G., Cano, R. J. & Rollo, F. 1998 Sequence analysis of bacterial DNA in the colon of an Andean mummy. *Am. J. Phys. Anthropol.* (In the press.)

Van de Peer, Y. & De Wachter, R. 1993 TREECON, a software package for the construction and drawing of evolutionary trees. *Comput. Appl. Biosci.* **9**, 177–182.

Vernesi, C., Caramelli, D., Carbonell i Sala, S., Ubaldi, M., Rollo, F. & Chiarelli, B. 1998 Application of DNA sex tests to bone specimens from three Etruscan (VII–III century BC) archaeological sites. *Ancient Biomolecules.* (In the press.)

Discussion

M. Spigelman (*University College London Medical School, UK*). Whilst the survival and finding of both human mitochondrial and ancient bacterial DNA can give a significant clue to whether there is present, in any particular specimen, the DNA of ancient pathogenic organisms, would you not agree that *M. tuberculosis*—with its ability to partly encapsulate, and the fact that it can be recovered from the soil up to one year after burial—gives it an ability to survive the effects of 'agonal septicaemia'. Also, it is protected from most of the early DNAses of the initial decomposers of the body, thus it may be that *M. tuberculosis* may be found in specimens that look less than promising on the above criteria.

F. Rollo. The point I intended to make with the exposition of those criteria is that a palaeomicrobiological investigation should not rely on the sole presentation of a sequence result. In the case of *M. tuberculosis,* for example, I believe one should anyway show how the pathogen DNA distributes in the different organs–tissues, and determines DNA copy number. If other support is also given, the confidence in the final result will be further strengthened.

R. P. Ambler (*University of Edinburgh, UK*). Would the procedure you used for isolation of DNA from mummy intestine or Ice Man muscle be sufficient to liberate DNA from ungerminated clostridial spores?

F. Rollo. We have not checked with modern spores. However, on the basis of what is known about ultrastructure degradation in archaeological specimens, I believe that DNA might be liberated from ancient spores using current protocols.