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The Isolation of *Bacillus amyloliquifaciens* and *B. astrophaeus* from Amber

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Abstract: Two bacteria (*Bacillus amyloliquifaciens* and *B. atrophaeus*), were isolated from Dominican amber using a novel isolation technique designed to avoid the possibility of contamination. A piece of amber seen to contain fungal hyphae was also cracked open under sterile conditions, but no fungal growth resulted. My findings are discussed in relation to the problems involved in isolating microorganisms from amber.

Key words: Survival of microorganisms, Ancient bacteria

INTRODUCTION

Amber has been shown to contain both fossilised and living bacteria and fungi (Lambert, *et al.*, 1998; Cano and Borucki, 1995; Cano and Borucki, 1997; Cano, 2003; Greenblatt, *et al.*, 2000). While it is claimed such isolates are as old as the amber itself (i.e. at least 25- 40 million years), not surprisingly, critics have suggested that they are modern contaminants (Bechanbach, 1995).

Here, I discuss experiments aimed at isolating microorganisms from Baltic and Dominican amber using an experimental approach designed to avoid all chance of contamination. The experiments were not designed to isolate all of the bacteria present in the amber samples used, nor to determine the frequency, or variety, of microorganisms in amber. Since two bacterial isolates were isolated from ancient amber, I took this as confirmation that amber contains bacteria and did not vary the media, or conditions used, in order to obtain further isolates.

MATERIALS AND METHODS

Nature the amber and confirmation of its authenticity:

Samples of Baltic amber, containing insect inclusions, were obtained from a variety of suppliers (Fig.1.). The authenticity of the individual amber samples was confirmed by the "salt water test" and by the fact that they emitted a pine resin odour when exposed to a hot wire or when scored with a serrated blade (Ross, 1998). The absence of surface cracks in the amber was confirmed using a hand lens and low power microscope.

In order to release the inclusions into media, the amber was cracked open and broken into small pieces using the vessel shown in Fig.2. This consisted of a thick glass-walled, tissue homogenising vessel, sealed with a metal cap. A plunger passed through the cap and touched the bottom of the vessel. The top of the cap was covered with part of an autoclave bag (using a corner cut from a large autoclave bag), attached and sealed closed using autoclave tape attached to the cap and the top of the plunger. The ability of the autoclave bag cover to act as an airtight seal and thereby prevented ingress of contaminants, was checked by inverting it under water and pumping air into it via the cracking vessel cap; absence of air bubbles confirmed the air tight nature of the seal. For the isolation of bacteria, Nutrient broth (Oxoid, 10mls) was added to the cracking vessel which was then autoclaved for 20mins at 120^0C.

Scoring and sterilisation of the amber:

A shallow central indentation was made in the surface of the amber and shallow groves were scored (with a serrated knife) from this indentation around the amber. The amber was immersed in domestic bleach (10% v/v) for 20 mins and

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Fig. 1: A typical piece of Baltic amber employed in these experiments

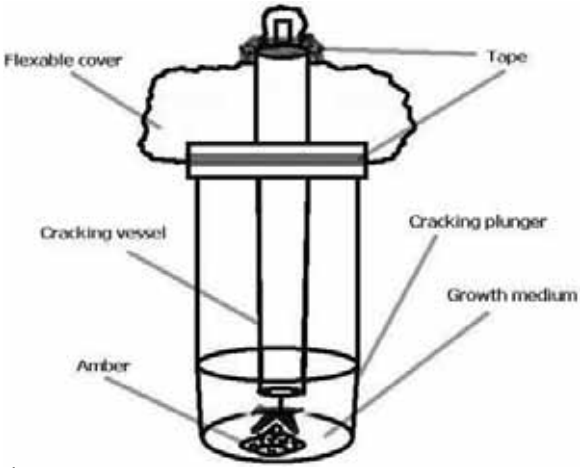

Fig. 2: The amber cracking vessel.

then transferred to a closed bottle containing sterile distilled water (500mls) and washed vigorously. The amber was then removed and immersed in membrane-filtered (0.22µm) alcohol and transferred to a flame, using flame–sterilised forceps; the residual alcohol was then ignited. The sterilised amber was finally transferred to the growth medium (10mls) in the cracking vessel.

Incubation of the vessel and isolation of bacteria:

After inserting the sterile amber, the cracking vessel was left for 4 days at 25^0C. All vessels in which bacteria grew in the medium were considered to be contaminated and therefore discarded. Where no bacterial growth appeared in the medium, the vessels were opened in a laminar air-flow cabinet and a small amount of medium was poured onto a Nutrient Agar (Oxoid) plate; this was then incubated at 25^0C for a further 4 days. If no bacteria appeared on the Nutrient Agar, or in the Nutrient Broth, over this time period the amber was cracked *in situ* in the vessel. If, following this period of incubation, bacterial growth appeared in the Nutrient Broth the vessel was opened, the neck of the vessel was thoroughly flame sterilised and a small amount of broth was aseptically transferred to Nutrient Agar; this was then incubated at 25^0C until growth appeared. Any bacterial isolates were then purified by streaking and were independently identified (NCIMB, Aberdeen) using 16SrRNA analysis. All transfers were done in a laminar air flow cabinet, the sterility of which was periodically checked.

Cracking of the amber:

The sterilised amber was cracked *in situ* in the cracking vessel by placing the bottom of the plunger into the surface indentation and applying a sharp tap to the top of the plunger with a light-weight hammer. The force cracked the amber along the scored groves to expose any fossilised insects inside. Further crushing of the amber was then achieved by manually applying force to the top of the plunger, until the original amber was crushed to form a suspension of very small particles.

Attempted isolation of a fungus from Dominican Amber:

An authenticated piece of Dominican amber containing a fungus was sterilised and cracked (as described above) in Czapek Dox liquid medium (Oxoid) containing 0.2g l^{-1} mycological peptone (Oxoid). The cracking vessel was incubated at 25^0C for 10 days when the contents were transferred to a sealed, sterile bottle (1litre) containing distilled water (500mls); the bottle was then incubated at 25^0C for a further 20 days.

Controls Although the experiments provide an "internal control" against contamination, 10 amber cracking vessels containing media were also exposed to the same handling procedures described above.

RESULTS AND DISCUSSION

Studies on the isolation of bacteria from Baltic amber:

Twenty different pieces of Baltic amber, containing unidentified insect inclusions (Fig. 1), were studied. After cracking, bacterial growth occurred in the medium in only one case; the media in the other 19 vessels remained clear. No growth occurred in any of the 10 amber cracking vessels that acted as controls. Two bacteria were isolated from the vessel in which growth occurred; these were independently identified by 16SrRNA analysis as *Bacillus amyoliquifaciens* or *B. atrophaeus* (these bacteria are too close to separate) and *B. cereus*. (Fig.3). Since the medium was checked for sterility prior to cracking and the tube remained sealed throughout the experiment, I conclude that these bacteria originated from within the amber. It could be argued that un-germinated spores were present on the surface of the amber prior to it being cracked. This could be the case, although plating prior to cracking showed that the medium itself was not contaminated in this way. The amber sterilisation technique was also checked by placing sterilised samples on Nutrient Agar; no bacterial growth appeared around the embedded amber. While the presence of a single contaminating spore, of each of the two bacteria, at some stage in the experiment can never be ruled out, the sterility checks used are rigorous as I can make them. The question of contamination naturally arises in studies such as these, where apparently ordinary, modern bacteria are isolated from ancient or highly extreme environments. The advantage of the approach used here is that the system contains and internal control, in that the amber is only cracked if the medium is seen to be uncontaminated; any bacterial growth following cracking of the sample must, as a result, have come from inside the amber.

Other workers have isolated bacteria from amber (Lambert, *et al*., 1998; Cano and Borucki, 1995; Cano and Borucki, 1997; Cano, 2003; Greenblatt, *et al*., 2000). Although no attempt was made here to demonstrate the antiquity of my isolates, others (by using genome analysis) have claimed that bacteria isolated from amber are millions of years old (Lambert, *et al*., 1998; Cano and Borucki, 1995; Cano and Borucki, 1997; Cano, 2003). Lambert *et al.,* (1998) for example, isolated a species of a *Staphylococcus* (*S. succinus*) from 25-40 million year Dominican amber while Cano and Borucki (Cano and Borucki, 1995; 1997; Cano, 2003) reported the isolation of *Bacillus sphaericus* (a species phylogenetically close to *B.amyoliquifaceans*); Greenblatt *et al*. (2000) have also isolated a wide range of bacteria from Dominican and 120 million year old amber from Israel. Although the authors of these studies discount the possibility of contamination, there work has nevertheless been criticised on this basis. Contamination can clearly never either be ruled out or be adequately controlled for. For example, it is possible that, despite a thorough microscope examination of the amber used here, a minute crack existed in the amber in which bacterial spores could reside, protected from the amber sterilisation process.

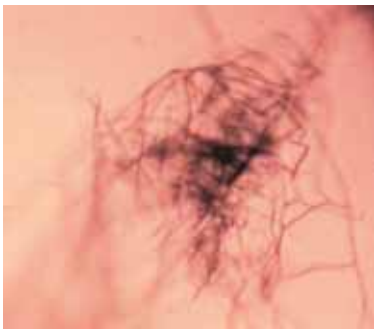

Fig. 3: Fungal hyphae inside amber.

Since the sample of amber that resulted in bacterial growth contained enclosed air bubbles, it is noteworthy that Ascaso *et al*., (2003) reported observing bacteria-like inclusions in amber air pockets. It may be that bacteria survive better in such air bubbles than they do in the insect inclusions; as a result amber samples containing such bubbles may be a more productive source of bacterial isolates than amber that is free of such inclusions.

It might be assumed that any microorganims isolated from amber must be able to grow under anaerobic conditions. However, survival, rather than growth, under anoxic conditions is the essential requirement. Since both of the bacteria isolated here are facultative anaerobes survival, in the anaerobic environment assumed to be present deep in amber, should not present a problem, at least in relation to this possible limitation.

Studies on the isolation of a fungus from Dominican amber:

Microscope examination(x10 magnification) of the sample of Dominican amber studied clearly shows the presence of a mass of fungus mycelium (Fig.3). The fungus occurs as a mass of very fine hyphae showing numerous anastomoses (i.e. connections), so as to from a web-like network. Such networks, or gossamers, are typically seen when fungi are grown under oligotrophic (i.e. low-nutrient) conditions. No fungi (or bacteria) were isolated in the original medium after 10 days incubation, or following dilution of the original medium in sterile water and a subsequent 28 day incubation period. The dilution step was introduced in case the fungus was inhibited by the original nutrient-rich medium and preferred instead to grow as an oligotroph. Fig. 3 shows that fungus was clearly present in the amber prior to cracking; this was presumably dead, or did not grow under the nutrient conditions imposed. It should be emphasised that only one attempt was made here to isolate fungus from amber; this compares with the finding that bacteria were isolated on only one occasion when 20 pieces of amber were cracked; clearly a fungus might be isolated should a large number of fungus-containing pieces of amber be sacrificed. It could also be argued thet the amber-fungus might have been induced to grow had a range of media been used. While this is true, only one sample of amber, containing a fungus, was available and the introduction of new medium into the cracking vessel would have fundamentally compromised sterility.

In view of the complexity of eukaryotic organisms, compared to bacteria, it is perhaps not surprising that fungi, even when present, are not readily grown from amber after entrapment for periods of millions of years. Despite this, Cano and Borucki (1997), claim to have isolated fungi, including a species of *Penicillium* from amber.

If the above contamination problems are discounted, I am then left to conclude that bacteria are preserved in amber and are resuscitated after at least 40 million years of stasis. Despite the known ability of spores of *Bacillus* species to survive over long periods (Driks, 1999) this possibility remains difficult to accept. However, set against this is the fact that bacteria, notably *Bacillus* species, have now been isolated, using a range of different experimental protocols, from geographically distinct amber samples. Added to this are reports of the isolation of *Bacillus* species from ancient salt crystals (Vreeland, *et al*., 2000). However, absolute proof of the existence of resusitatable bacteria in 40 million year old amber will presumably have to await an experimental approach that allows for the detection of viable bacteria within the amber itself.

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